Molecular Effects of Polyphenols in Experimental Type 2 Diabetes Mellitus and Metabolic Syndrome

DISSERTATION

zur Erlangung

des naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg

vorgelegt von

Stoyan Dinkov Dirimanov

aus Sofia

Würzburg

2019

Eingereicht bei der Fakultät für Chemie und Pharmazie am: .....

Gutachter der schriftlichen Arbeit:

1. Gutachter: .....

2. Gutachter: .....

Prüfer des öffentlichen Promotionskolloquiums:

1. Prüfer: .....

2. Prüfer: .....

3. Prüfer: .....

Datum des öffentlichen Promotionskolloquiums:

.....

Doktorurkunde ausgehändigt am:

.....

Die vorliegende Arbeit wurde auf Anregung von

Frau Professor Dr. Petra Högger

am Lehrstuhl für Pharmazeutische Chemie

des Instituts für Pharmazie und Lebensmittelchemie

der Julius-Maximilians-Universität Würzburg angefertigt.

Meiner Eltern

Poster Presentations

Dirimanov S., Xiao J., Högger P.
Role of polyphenols in vascular health
ISPMF 2015, 2nd International Symposium on Phytochemicals in Medicine and Food, June 26-29, 2015, Shanghai, China

Dirimanov S., Xiao J., Högger P.
The protective role of polyphenols in diabetes related vascular complications
ICNPU 2015, 2nd International Conference on Natural Products Utilization: from Plant to Pharmacy Shelf, October 14-17, 2015, Plovdiv, Bulgaria

„Nicht durch die kraft höhlet der tropfen den stein,
sondern durch häufiges fallen.”

„Gutta cavat lapidem, non vi, sed saepe cadendo.”

Ovid

Acknowledgments

First of all, I would like to express my sincere gratitude to Prof. Dr. Petra Högger for accepting me as a doctoral student and giving me the opportunity to get involved in a project regarding intriguing and socially significant topic. In addition, I am very grateful for her awesome supervision, original ideas, understanding and readiness to discuss openly every challenge that occurred during the project.

I would like to thank Prof. Ing. Zdeňka Ďuračková (Comenius University in Bratislava) and the Horphag Research for organizing and carrying out the clinical study and for kindly providing volunteers' samples for the present investigations.

Special thanks are due to Prof. Dr. Christoph Sotriffer for the opportunity to collaborate with his working group and to learn a lot about *in silico* models. I am very grateful to Mr. Mathias Diebold for his time, support, discussions, and also for supplying me with several compound databases for virtual screenings.

I would like to gratefully acknowledge the organizations BAYHOST and FAZIT-Stiftung for providing me a financial support, and thus making the completion of this work possible. Furthermore, I would like to thank the persons who positively assessed my progress and supported me for obtaining and prolonging my funding: Prof. Dr. Petra Högger, Prof. Dr. Michael Decker, and PD Dr. med. Asparouh Iliev.

Moreover, I would like to thank to:

- The present and former members from the working groups of Prof. Högger (Roswitha Scheblein, Dr. Frederic Vollmers, Dr. Martin Haas, Dr. Melanie Mülek, Dr. Steffen Jeßberger, Dr. Maike Scherf-Clavel, Dr. Jianbo Xiao, Dr. Hui Cao, Dr. Lisa Schaaf, Andreas Pospiech, Linda Volpp, Florian Lang, Charlotte Beier, Theresa Häfner, Arthur Felker, Jasmin Bayer) and Prof. Scherf-Clavel (Prof. Dr. Oliver Scherf-Clavel, Bettina Friedl, Sebastian Zimmermann, Maximilian Stapf) for the positive attitude, friendship, and helpfulness. Danke für alles!
- Prof. Dr. Jianbo Xiao for providing me the opportunity to present parts of my work on the ISPMF 2015 conference in China
- The colleagues from the Chair for Drug Formulation and Delivery (working group Prof. Meinel) who readily shared their equipment and discussed intriguing scientific issues with me, especially: Dr. Marcus Gutmann, Dr. Gabriel Jones, Dr. Fang Wu, and Dr. Alexandra Braun.
- My colleagues for the pleasant time during the teaching activities (5. Semester): Theresa, Christine, Klaus, Daniela, Maike, Melanie, Felix, David and Frederic.
- Dr. Stefan Ivanov, Vania Dirimanova and Eng. Liubomir Bambov who checked some parts of the present thesis for spelling. Special thanks to Stefan for our talks and his expert opinion regarding a variety of scientific topics.

Furthermore, I am very thankful to my family and friends who supported me and encouraged me all the time. Last but not least, I would like to thank you Giovanna for staying by my side and for sharing every single moment of sadness and joy during the years of my study.

Table of Contents

A Introduction

1 Diabetes mellitus

1.1 Background

Diabetes mellitus is characterized by chronically increased blood glucose levels due to absolute or relative deficiency of insulin. It is highly prevalent and along with its complications it is among the leading causes of death. Presently, according to the latest global report on diabetes of the World Health Organization (WHO), in 2014, approximately 8.5 % of people worldwide were diabetics. This number was 4.7 % in 1980, indicating a worrying trend of increasing morbidity over the last three decades [1]. Recent data (2015) from the American Diabetes Association show that diabetes prevalence among adults in the U.S. is 9.4 % [2], while in the European Union it was reported that 6.9 % of the population (2014) suffer from this disease [3]. Diabetes is considered a socially significant morbidity because of its considerable negative impact on the patients' duration and quality of life. Therefore, research activities are focused on developing novel and more effective therapies, aiming to improve glucose homeostasis and alleviate insulin resistance.

Two main types of diabetes according to its pathogenesis can be distinguished – diabetes mellitus type 1 (T1DM) and type 2 (T2DM). However, novel diabetic subgroups based on additional factors for the disease's occurrence, such as body-mass index, age, β-cell function, insulin resistance, and presence of autoantibodies aim at a more precise and individualized therapeutic handling [4].

1.2 Types of diabetes

1.2.1 Type 1 diabetes

Type 1 diabetes mellitus (T1DM) typically has a sudden onset and is caused by a pancreas dysfunction resulting in insufficient or completely abolished insulin production and secretion. Common symptoms are frequent urination (polyuria), excessive thirstiness (polydipsia), hunger (polyphagia), fatigue, blurry vision, unexpected weight loss, and delayed wound healing. This form of diabetes is caused by autoimmune processes targeting insulin producing β-cells [5]. It usually occurs at a young age. However, a form of T1DM manifesting later in adulthood also exists – latent autoimmune diabetes of adults (LADA) [5]. It can be falsely diagnosed as T2DM. The risk of short- and long-term complications necessitates tight blood glucose control. Novel medical devices which have

recently been approved or are undergoing clinical trials may contribute to improving diabetics' quality of life. Such devices are insulin pumps, closed-loop glucose monitoring systems, and artificial bionic pancreases [6]. Type 1 diabetes is managed by insulin replacement treatment.

1.2.2 Type 2 diabetes

Type 2 diabetes mellitus (T2DM) is commonly described as a lifestyle-related condition. It represents around 95 % of all diabetes cases [7]. As the symptoms of T2DM are not specific, it can remain latent and undiagnosed over a long period of time. Obesity and increased insulin levels are forewarnings of insulin resistance. A chronic pro-inflammatory state is present as well. Aging, oversupply of nutrients, and lack of physical activity are key factors in diabetes pathogenesis and development of insulin resistance. In order to compensate for the latter, β-cells are continuously stimulated to produce insulin and in spite of high blood levels (hyperinsulinemia) increased postprandial glucose levels are not normalized. As a result of the permanent stress, β-cells deteriorate and may undergo apoptosis. Therefore, in the advanced stage of uncontrolled T2DM, insulin production can be insufficient and patients require insulin treatment. Being a complex disorder, T2DM is often accompanied with metabolic syndrome including insulin resistance, obesity, dyslipidemia, and hypertension [5, 7].

1.2.3 Further types of diabetes

Gestational diabetes is characterized by increased blood glucose levels occurring in healthy pregnant women due to insulin resistance, likely caused by certain pregnancy-related hormones. Gestational diabetes usually resolves after childbirth. It represents a very little risk for the mother (rarely development of T2DM), but a huge threat for the baby, as it can contribute to a number of negative consequences, such as postnatal low blood glucose, increased body size, and disrupted organogenesis. Thus, this condition should be managed properly [7].

Maturity onset diabetes of the young (MODY) is a very rare form of diabetes mellitus different from T1DM and T2DM. It is caused by single gene mutations related to insulin production and can be inherited [7].

Steroid diabetes may occur due to long-term use of corticosteroids, which antagonize insulin's action and may cause increase in blood sugar levels [7].

1.3 Prediabetes

Prediabetes (borderline diabetes) is a transitional pathological state which is characterized by increased blood glucose levels, which are not high enough for diabetes to be diagnosed [1]. It may frequently, but not always advance to T2DM. Prediabetes can be subdivided

into two types. Impaired fasting glucose (IFG) is characterized by permanently increased fasting glucose levels. Impaired glucose tolerance (IGT) can be described as a failure of the organism to restore its blood sugar to normal concentrations within two hours after an oral challenge of 75 g glucose (oral glucose tolerance test – OGTT). Hyperglycemia is a hallmark sign of a metabolic disorder. Therefore, glucose levels are of both pathophysiological and diagnostic importance and can provide a hint as to the progress and state of diabetes (Table 1).

Table 1.: Referent blood glucose concentrations as diagnostic criteria for prediabetes and diabetes mellitus state and progression according to the World Health Organization (WHO) [1]:

Pathological state	Blood glucose levels	
	mg/dL	mmol/L
Diabetes		
Fasting plasma glucose	≥ 126	≥ 7.0
2 h post-challenge plasma glucose*	≥ 200	≥ 11.1
Impaired fasting glucose (IFG)		
Fasting plasma glucose	110 – 125	6.1 – 6.9
(2 h post-challenge plasma glucose*)	(< 140)	(< 7.8)
Impaired glucose tolerance (IGT)		
Fasting plasma glucose	< 126	< 7.0
2 h post-challenge plasma glucose*	140 – 200	7.8 – 11.1

* 75 g oral glucose load

Both forms of prediabetes are related to insulin resistance and might be coupled to other aspects of metabolic syndrome. Hyperglycemia plays a major role in short and long term diabetes complications' pathogenesis. Thus, prediabetes correlates with an increased risk of occurrence of cardiovascular complications [8]. Therefore, duly lifestyle changes for persons with prediabetes are strongly recommended.

1.4 Insulin resistance

Insulin resistance can be described as an impaired cellular response to insulin, which leads to hyperglycemia. As a result of the increased blood glucose levels, insulin production by the pancreas is stimulated, resulting in hyperinsulinemia. Obesity and increased free fatty acid levels are discussed as important factors for insulin resistance [9]. Certain adipokines and pro-inflammatory cytokines, such as tumor necrosis factor α secreted by adipocytes reduce insulin sensitivity [9]. A widely used test for quantification of insulin resistance is

homeostatic model assessment of insulin resistance (HOMA), in which both fasting glucose and insulin serum concentrations are considered [10].

Insulin effects at the molecular level are mediated mainly through two signaling cascades: PI3K/Akt (A-2.1) and MAPK (A-2.3). An imbalance between these pathways in the insulin resistance state occurs: a prevalence of MAPK signaling over PI3K/Akt [11]. This phenomenon has been demonstrated in a human study involving obese non-diabetics and T2DM-patients compared to the healthy participants in the control arm [12]. PI3K/Akt is related to vasoprotection (mediated by nitric oxide) and glucose uptake. MAPK provokes vasoconstriction (through endothelin-1), inflammatory response, vascular smooth muscle proliferation, and inflammatory response [11]. This can explain at least in part the relationship between insulin resistance, endothelial dysfunction, and the cardiovascular components of metabolic syndrome.

1.5 Endothelial dysfunction and vascular complications

The endothelium is a thin cellular layer on the internal surface of blood vessels, exercising a key regulatory role in the cardiovascular system, being responsible for vascular tone, remodeling, and angiogenesis [13]. The endothelium integrates endocrine, paracrine and autocrine signals in order to maintain cardiovascular homeostasis. Endothelial dysfunction (ED) is characterized by a loss of certain functions by endothelial cells. This involves an altered balance between vasodilators and vasoconstrictors, leading to impaired endothelial-dependent vasomotility and a disrupted barrier function. It is frequently accompanied by pro-inflammatory and pro-thrombotic states [13]. In T2DM major factors involved in the development of ED are aging, oxidative stress, hyperglycemia, dyslipidemia, and the presence of insulin resistance (A-1.4) [11, 13], while in T1DM hyperglycemia plays the primary role. Compromised endothelial function can precede atherosclerosis and is a prerequisite for the emergence of cardiovascular complications such as microangiopathy (retinopathy, nephropathy) macroangiopathy (myocardial infarction, stroke), and diabetic cardiomyopathy [11, 13].

1.6 Management of T2DM

T2DM is discussed to be a largely preventable disease. Nevertheless, morbidity and mortality rates, especially in low- and middle-income countries, remain high. The International Diabetes Federation offers guidelines for non-pharmacological and pharmacological management of T2DM [14]. Lifestyle changes, i.e. restricted caloric intake and physical exercise, are recommended. Recent clinical studies showed that nutritional improvements can be a promising strategy in T2DM treatment [15, 16]. An open-label, cluster-randomized human trial (DiRECT) involving 306 diabetics, who had been diagnosed within the past six years [15], revealed that a 12 months low-calorie

dietary intervention induced diabetes remission in almost half of the participants. Furthermore, around 90 % of the individuals with weight loss of 15 kg or more achieved remission [15].

International Diabetes Federation guidelines also describe diabetes treatment with approved drugs [14]. Metformin is proposed as first-line therapy. Alternatively, sulfonylureas (possibly with low risk for hypoglycemia) or dipeptidyl peptidase 4 inhibitors can be prescribed. A detailed recent review by Marín-Peñalver et al. is focused on diabetes pharmacotherapy [17], which is outside the scope of the present work. Herein, a brief overview of certain approved drugs and their molecular effects on two signaling pathways with high relevance to diabetes: Akt (A-2.1) and AMPK (A-2.2), is presented (Table 2).

Table 2.: List of certain approved drugs shown to have effects on the diabetes relevant signaling pathways Akt and AMPK. The data does not originate from human studies, but from *in vitro* (*) and *in vivo* (**) models.

Drug	Therapeutic class	Experimental effects on			
		Akt		AMPK	
Metformin	Biguanidine	Inhibition [18]	*,**	Activation [19]	*
Insulin	Peptide hormone	Activation [20]	*,**	Inhibition [21]	*
Rosiglitazone	Insulin sensitizer	Activation [22]	**	Activation [22]	**
Exenatide	Insulin secretagogue	Activation [23] Inhibition [24]	* *	Activation [23]	*
Sitagliptin	Dipeptidyl peptidase IV inhibitor	Activation [25]	**	Activation [25]	**
Canagliflozin	Sodium glucose co-transporter-2 inhibitor	Inhibition [26]	*	Activation [27]	*,**

2 Diabetes and cell signaling

2.1 PI3K/Akt

PI3K/Akt is a key signaling pathway responsible for fundamental cellular processes, such as protein synthesis, glucose uptake and metabolism, proliferation and cell survival [28]. This pathway mediates the insulin metabolic effects on the cellular level and includes a cascade of kinases: insulin receptor, insulin receptor substrates, phosphoinositide-3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK1), and protein kinase B (Akt/PKB, Figure 1).

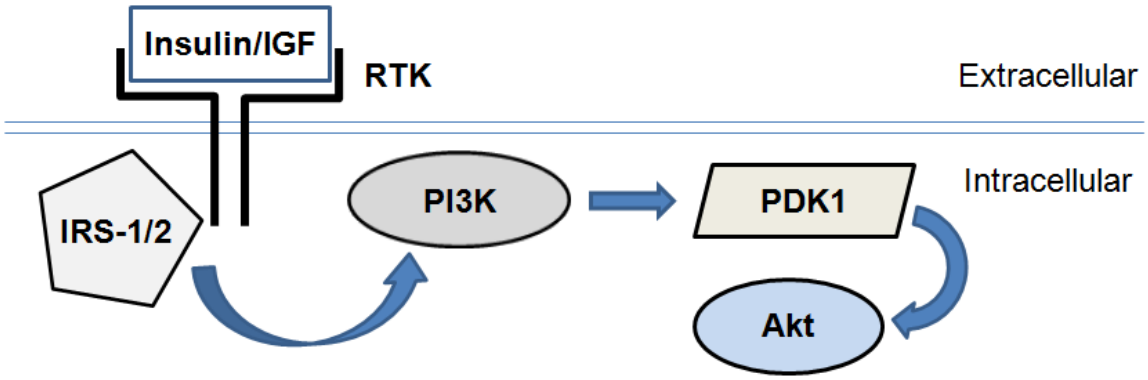


Figure 1: Simplified scheme of PI3K/Akt signaling pathway. Peptide ligands, such as insulin or insulin-like growth factor-1 (IGF) can bind and activate receptor tyrosine kinases (RTK; i.e. insulin receptor). In turn it further activates a cascade of several enzymes: insulin receptor substrate-1/2 (IRS-1/2), phosphoinositide-3-kinase (PI3K), phospho-inositide-dependent kinase 1 (PDK1) and Akt/PKB.

PI3K activation is triggered by receptor tyrosine kinases (RTKs) such as the insulin receptor or by G-protein coupled receptors, e.g. angiotensin II receptor. In response, PI3K produces a lipid second messenger PIP3 (phosphatidylinositol-(3,4,5)-trisphosphate) which activates downstream pathways like Akt or atypical protein kinase C [29] (Figure 2).

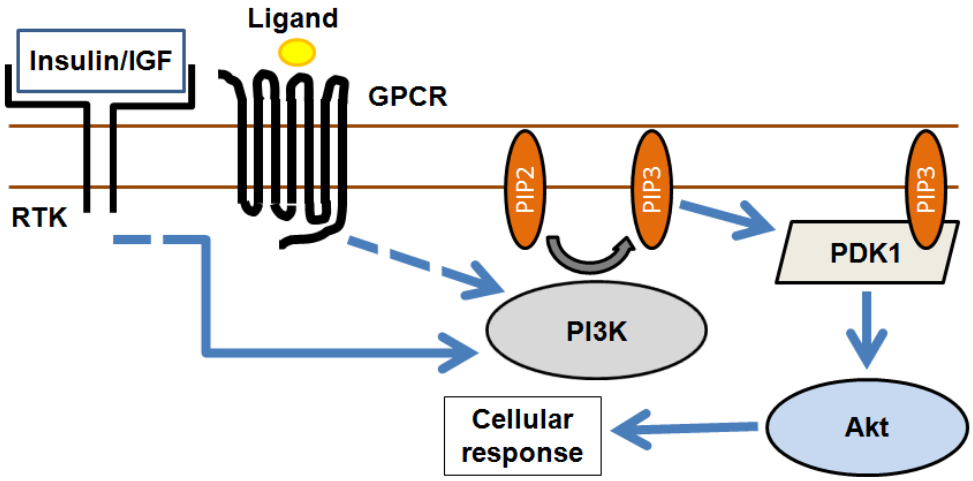


Figure 2: Different stimuli from receptor tyrosine kinase (RTK) or G-protein coupled receptor (GPCR) can trigger an activation of phosphoinositide-3-kinase (PI3K). In turn, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) and produces a lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which in turn activates downstream kinases such as PDK1 and Akt/PKB, involved in regulation of diverse cellular processes.

2.1.1 Protein structure of Akt kinase

Akt is a serine/threonine protein kinase from the AGC kinase family (AMP/GMP-kinases and PKC), indicating its homology with protein kinases A and C. Akt is composed of three main conserved domains (Figure 3): an N-terminal pleckstrin homology domain, a central catalytic domain, and a C-terminal extension.

Figure 3: Schematic illustration of the Akt/PKB protein structure. Main structural domains are the pleckstrin homology domain (PH) linked to the highly conserved central catalytic kinase domain (CAT) through the linker region (LINK), and the C-terminal extension (EXT). The most important phosphorylation sites – serine (Ser473) and threonine (Thr308) – are shown as well; numbers indicate amino acid position in the Akt kinase sequence.

In addition, a short linker region connects the pleckstrin homology domain and the central catalytic one [30]. Each of these regions has distinct functions. The pleckstrin homology domain is responsible for binding of Akt to second messengers (phosphatidylinositols) and

for trafficking of the protein to different membrane compartments. Interactions between the pleckstrin homology domain and phospholipids are charge-charge driven. The central catalytic domain includes sites important for kinase activity: the ATP-binding site and the substrate-binding site. A hydrophobic motif belongs to the C-terminal extension and has regulatory functions for kinase activity.

2.1.2 Akt isoforms

Presently three isoforms of Akt are known: Akt1, Akt2, and Akt3 (also named PKBα, PKBβ, and PKBγ). They are structurally similar, but functionally different. The conserved pleckstrin homology-, central catalytic-, and C-terminal extension-domains are highly similar for all three isoforms. In contrast, the linker region displays the highest diversity among them [30]. Akt1 is ubiquitously expressed within the body and is responsible for cell survival and growth. Akt2 can be found in target tissues of insulin (fat and muscles). This isoform conveys the effect of insulin on glucose transport through glucose transporter 4 (GLUT4) and contributes to cellular metabolism. Akt3 is expressed in the brain [28]. The functionality of the isoforms has been confirmed by studies with knockout animals. Deletion of the *Akt1* gene caused a delay in growth and body weight decrease. *Akt2* deletion in turn caused impaired blood glucose control. None of these single gene deletions was lethal. In contrast, simultaneous silencing of both *Akt1* and *Akt2* resulted in neonatal death. Thus, the importance of Akt-signaling was demonstrated; it has been suggested that both isoforms have distinct functions, but they are interchangeable and might partially compensate for each other in the absence of one of them [31]. The experimentally established role of Akt for insulin sensitivity was confirmed in a human study. A dominant *Akt2* mutation resulting in the expression of a nonfunctional kinase was responsible for the development of insulin resistance and severe diabetes in humans [32].

2.1.3 Regulation of Akt activity

Akt kinase can be activated through phosphorylation at a threonine (Thr308) site at the activation T-loop close to the catalytic core or at a serine (Ser473) residue of the hydrophobic motif of the C-terminal extension (Thr309 and Ser474 for Akt2, respectively). These post-translational modifications lead to conformational changes of the kinase structure ensuring access for both ATP and substrate to the catalytic center and thus increasing the enzyme's activity. In general, insulin- or growth factor- induced Akt activation is governed by PI3K. The direct upstream kinase responsible for Thr308 phosphorylation of Akt is phosphoinositide-dependent kinase 1 (PDK1), while Ser473 phosphorylation can be primarily achieved through the mTORC2 (mechanistic target of rapamycin complex 2) pathway [33]. The produced from PI3K phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) interacts with the pleckstrin homology domain of Akt resulting in its recruitment to the plasma membrane. The importance of the hydrophobic motif- [34] and pleckstrin homology-domains [35] for the protein-protein interaction

between Akt and its upstream PDK1 needed for the Thr308 phosphorylation has been demonstrated. Several studies show that the kinase responsible for Ser473 phosphorylation – mTORC2 – can be activated in both a PI3K-dependent [36] and independent manner [37].

Alternatively, Akt can be phosphorylated in a PDK1-independent manner by integrin-linked kinase-1, Ca^{2+}/calmodulin-dependent kinase kinase or protein kinase A [34]. Non-canonical activation of Akt can be achieved through phosphorylation by different non-receptor tyrosine kinases such as activated CDC42 kinase 1 [38] and protein tyrosine kinase 6 [39]. A novel activation mechanism involving phosphorylation at Ser477 and Thr479 at the extreme C-terminus of Akt by cyclin-dependent kinase 2 under special conditions has been recently discovered [40]. In addition, various potential phosphorylation positions were characterized by phosphorylation site mapping [41]. Further post-translational modifications shown to regulate Akt activity are acetylation, glycosylation in the catalytic center, and ubiquitilation [28].

In order to adjust the duration and strength of insulin-triggered signals, negative regulators of PI3K/Akt exist. They can be classified in two main groups: lipid phosphatases and protein phosphatases. The first group includes phosphatase and tensin homolog (PTEN) which directs the second messenger PIP3 and dephosphorylates it, thus preventing recruitment of Akt to membranes and its activation [42]. Protein phosphatases such as protein phosphatase 2A directly dephosphorylate the Akt protein [43]. These enzymes antagonize PI3K/Akt signaling. Therefore, decreased expression or activity of these phosphatases is associated with over-activation of this pro-survival pathway and malignancies [42].

In addition to upstream kinases and phosphatases, Akt activity is regulated through feedback loop mechanisms. Some of the downstream targets of Akt are capable of negatively regulating Akt phosphorylation through influencing on upstream kinases, thus dynamically adjusting the signal transduction process. For instance, Akt's downstream targets mechanistic target of rapamycin complex 1 (mTORC1) and ribosomal protein S6 kinase beta-1 (S6K1) were shown to deactivate insulin receptor substrate 1 through phosphorylation, further inducing its degradation [28]. As insulin receptor substrate 1 is upstream of PI3K and Akt, this decreases their activity. This negative feedback loop can contribute to insulin resistance [44, 45]. In addition, prolonged inhibition of the PI3K/Akt pathway led to up-regulated expression of receptor tyrosine kinases and enhanced susceptibility to insulin and growth factors. This feedback regulation was mediated by other Akt substrates: forkhead box O family transcription factors (FoxO) [46]. Thus, sustained activation or inhibition of PI3K/Akt might contribute to a manifestation of the opposite effect.

The Akt signaling network is further complicated by cross-talk between Akt and other key signaling pathways such as AMPK (5'adenosine monophosphate-activated protein kinase) and MAPK (mitogen-activated protein kinase) (see A-2.2 and A-2.3). Akt signaling is responsible for the cellular effects of insulin. Therefore, this pathway promotes anabolic processes. Conversely, AMPK is activated under energy supply restriction conditions and stimulates mainly catabolic responses. In this regard, both kinases act in opposing ways. Indeed, it has been demonstrated that Akt and AMPK are often regulated in a coordinated reciprocal manner [47]. Akt has been shown to directly prevent liver kinase B1-induced AMPK-activation [48]. A relationship between Akt and MAPK (especially extracellular signal-regulated kinases/ERK) signaling was demonstrated, as pharmacological inhibition of one of these pathways often resulted in the activation of the other [49]. Predominance of ERK over Akt signaling has been observed in insulin resistant states (see A-1.4). Akt, AMPK and MAPK have various common substrates. Despite their general counteracting roles in some cases, these kinases might act synergistically.

Insulin has differential effects on the subcellular distribution of Akt1 and Akt2, which indicates distinct physiological functions for the two isoforms. Akt2 showed sustained accumulation in the plasma membrane compartment to a greater extent than Akt1. This correlates with the specific role of Akt2 in the regulation of GLUT4 trafficking and insulin-mediated glucose transport [50].

The key role of Akt in the pathogenesis of T2DM stimulates research for further elucidation of its regulatory mechanisms as a base for the development of novel therapeutic strategies. Thus, Chakraborty et al. described the influence of endogenous inositol pyrophosphate on Akt signaling and suggested its role in insulin resistance. Inositol pyrophosphate competes with PIP3 for the binding site of pleckstrin homology domain and in this way prevents the recruitment of Akt to the plasma membrane and, therefore, reduces its phosphorylation by PDK1. As a result, a decrease in insulin-guided glucose uptake in skeletal muscles and fat cells has been observed [51]. The authors described inositol pyrophosphate as a physiological inhibitor of Akt. A targeted inhibition of inositol hexakisphosphate kinase-1, responsible for inositol pyrophosphate production, was suggested as a potential mechanism for alleviation of insulin resistance.

2.1.4 Akt substrates

The tight regulation of Akt suggests its pivotal place in governing essential physiological processes. In regard to this, Akt is a serine/threonine kinase capable of phosphorylating multiple substrates, implementing its cellular effects: glucose uptake and metabolism, protein synthesis, cell survival, and cardiovascular effects. Importantly, Akt (especially Akt2) has a fundamental role in carbohydrate metabolism. Akt phosphorylates a protein named 160 kDa Akt substrate (AS160, also known as Rab-GTPase-activating protein), which in turn controls the trafficking of the glucose transporter GLUT4 from vesicles in

the cytosol to the cell surface. Thus, in response to insulin stimulation, Akt intensifies the glucose uptake into cells [52]. Another main function of Akt is a regulation of glycogen synthase kinase-3β (GSK-3β) activity through a phosphorylation event having an inactivating effect. GSK-3β phosphorylates and, thus, inhibits the enzyme activity of glycogen synthase [53]. Therefore, activation of Akt leads to increased glycogen synthesis, mediating insulin-stimulated storage of excess glucose. This determines the relevance of GSK-3β in T2DM. Indeed, pharmacological inhibition of this kinase alleviated insulin resistance and reduced glucose levels in an *in vivo* diabetic model [54]. In addition to glycogen synthesis, GSK-3β is involved in regulation of multiple signaling pathways (e.g. Wnt) and gene expression [53]. A graphic overview of the Akt signaling network is shown on the Figure 4.

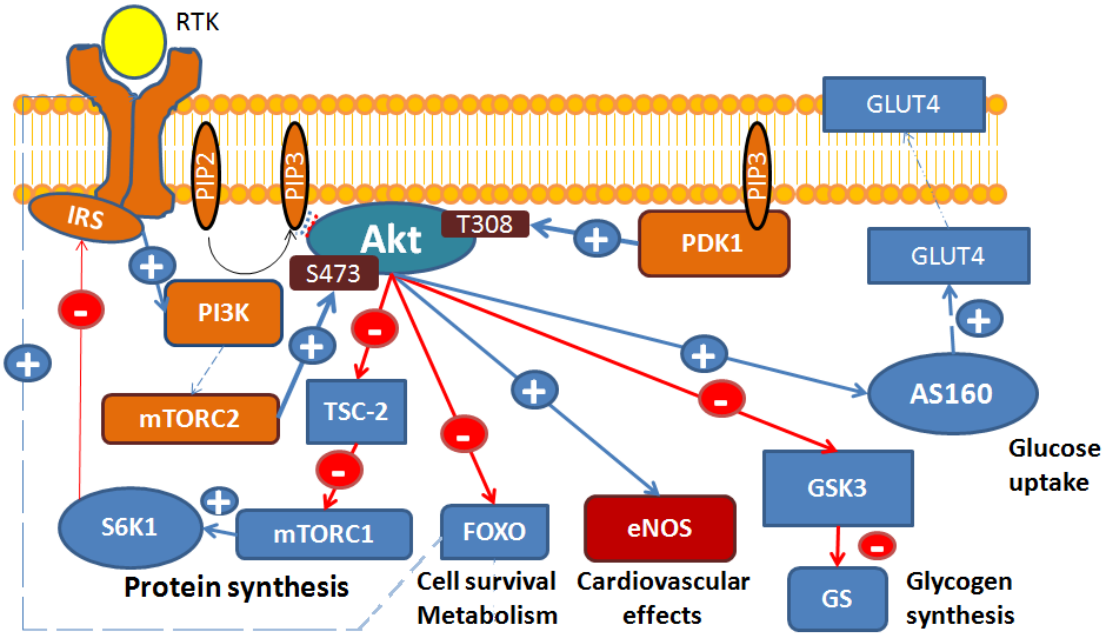


Figure 4: Akt signaling network – an overview. The activated receptor tyrosine kinase (RTK) activates insulin receptor substrate (IRS) and phosphoinositide 3-kinase (PI3K) promoting a second messenger production – phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Subsequently activated phosphoinositide-dependent kinase 1 (PDK1) phosphorylates Akt at a threonine 308 residue (T308). mTORC2 is responsible for a serine 473 site phosphorylation (S473). In turn, Akt phosphorylates following substrates: 160 kDa Akt substrate (AS160) regulating glucose transporter 4 (GLUT4), glycogen synthase kinase 3 (GSK3), endothelial nitric oxide synthase (eNOS), forkhead box O family transcription factors (FOXO), tuberous sclerosis complex 2/ mechanistic target of rapamycin complex 1/ ribosomal protein S6 kinase beta-1 pathway (TSC-2/mTORC1/S6K1). Feedback control mechanisms: the downstream target of Akt S6K1 inhibits upstream IRS; Prolonged inhibition of FOXO leads to up-regulation of RTK expression.

The fundamental role of Akt in protein synthesis is mediated by phosphorylation and inactivation of tuberous sclerosis complex 2 (TSC-2), which in turn is a physiological inhibitor of mTORC1. Thus, as a consequence of Akt-activation, mTORC1 becomes activated as well [55]. This kinase protein complex activates S6K and inactivates the mRNA translation repressor called eukaryotic translation initiation factor 4E-binding protein 1. These changes regulate positively initiation and translation which results in increased protein synthesis [56]. Chronic hyper-activation of mTORC1 induced by excessive nutrition was shown to contribute to insulin resistance and obesity [45].

Further substrates regulated by Akt are forkhead box O family transcription factors (FoxO). The PI3K/Akt pathway negatively regulates these transcription factors and enables their translocation outside of the nucleus and thus inactivates them. FoxO are responsible for regulating the expression of genes related to the induction of apoptosis, cell cycle arrest and metabolic changes [57]. It has been shown that *FoxO1* gene knockout reverses insulin insensitivity caused by liver *Akt1* and *Akt2* deletion in an animal model, suggesting their importance for T2DM pathophysiology [58].

2.1.5 Akt and the cardiovascular system

Akt kinase plays a vital role in the cardiovascular system. In addition to its pro-survival effects, Akt is involved in angiogenesis, vasorelaxation and vascular remodeling processes [59]. Potent activators of the Akt cascade are VEGF (vascular endothelial growth factor), PDGF (platelet derived growth factor) and insulin [60]. Subsequently, Akt targets a protein with an essential role for cardiovascular homeostasis maintenance: endothelial nitric oxide synthase (eNOS, NOSIII). This enzyme is widely expressed in endothelial cells and is responsible for the production of a gaseous signaling molecule – nitric oxide (NO), which diffuses towards vascular smooth muscles. It exhibits a plethora of effects such as vasorelaxant, angiogenetic, antithrombotic, anti-adhesive [61]. The main place of Akt in regulation of this pathway, known as PI3K/Akt/eNOS, defines its importance in cardiovascular pathologies such as diabetes-related complications.

It has been shown that hyperglycemia impairs the insulin triggered PI3K/Akt/eNOS activation [62]. This prevention of eNOS activation leads to a decrease in NO bioavailability which is considered to be the main mechanism in the development of diabetic micro- and macro-angiopathies [63].

2.1.6 Akt inhibitors

Inhibitors of Akt are comprehensively investigated as this kinase exhibits pro-survival and proliferation promoting activities and is overexpressed and/or over-activated in many human malignancies. Different strategies for a suppression of the Akt function have been employed. Based on their mode-of-action, Akt inhibitors can be divided in the following groups, targeting: the upstream effectors of Akt, Akt's ATP-binding site, pleckstrin homology domain, substrate binding site, and allosteric inhibitors, capable of selective isoform inhibition [30]. Various specific and potent Akt inhibitors were not only used in the preclinical research as pharmacological tools, but currently undergo clinical trials, e.g.: miransertib (ARQ-092), ipatasertib (GDC-0068), and capivasertib (AZD5363), (Figure 5) [64].

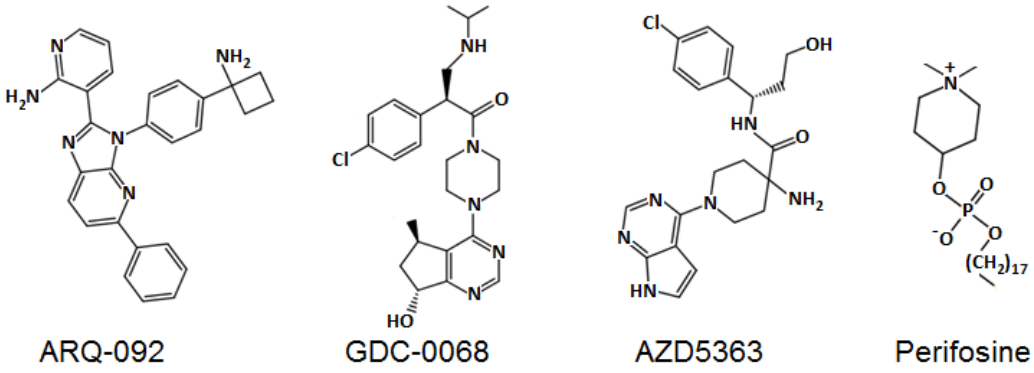


Figure 5: Chemical structures of certain Akt inhibitors undergoing clinical development: ARQ-092 (allosteric inhibitor), GDC-0068, AZD5363 (ATP-competitive inhibitors) and perifosine (pleckstrin homology domain inhibitor).

Challenges in the clinical development of Akt inhibitors are related to solubility of compounds, respectively their pharmacokinetic characteristics, and on-target toxicities, such as hyperglycemia. In addition, a special issue is the so-called rebound effect, manifesting in robust over-activation of the PI3K pathway caused by Akt inhibition and mediated through feedback mechanisms. This results in non-efficiency of single inhibitors alone and demands the application of combined therapies [28].

As the PI3K/Akt pathway is involved in pathogenesis of various human diseases different than cancer, it would be conceivable Akt inhibitors to be investigated for treatment of other pathologies. For instance, investigations to uncover the role of Akt inhibitors in the treatment of cardiovascular diseases are needed [59].

2.2 AMPK

AMPK (5'adenosine monophosphate-activated protein kinase) is known to be the cellular metabolic master switch which determines its pivotal role in T2DM. AMPK regulates glucose and fatty acids uptake, mitochondrial β-oxidation, and glycogen synthesis. Recent studies revealed that AMPK-signaling is dysregulated in T2DM [65, 66], and it has been proposed that its stimulation can be advantageous in diabetes and its related co-morbidities like obesity and metabolic syndrome because of an improvement of glucose homeostasis and insulin sensitivity.

2.2.1 AMPK – protein structure and regulation

This kinase is a conserved and ubiquitously expressed heterotrimer composed of one catalytic (α) and two regulatory (β and γ) subunits. Binding of AMP to γ causes conformational changes of the protein complex resulting in activation of AMPK through phosphorylation of Thr172 of the α catalytic subunit by the upstream liver kinase B1. Conversely, binding of ATP to γ leads to a decrease in AMPK activity. Furthermore, ADP-binding to γ leads to protected and sustained phosphorylation [67]. Apart from liver kinase B1, further upstream regulators able to activate AMPK are transforming growth factor-β activated protein kinase-1 and Ca^{2+}/calmodulin-dependent protein kinase kinase β [66]. As a response of increased calcium intracellular levels, the latter kinase can phosphorylate AMPK which is a mechanism independent from AMP/ATP- and ADP/ATP-ratios [68].

Various stimuli of a different nature are able to activate AMPK (i.e. Table 2 in A-1.6). They can be subdivided in the following categories: physiological, hormonal, naturally-originating, and pharmacological activators [66] (Figure 6).

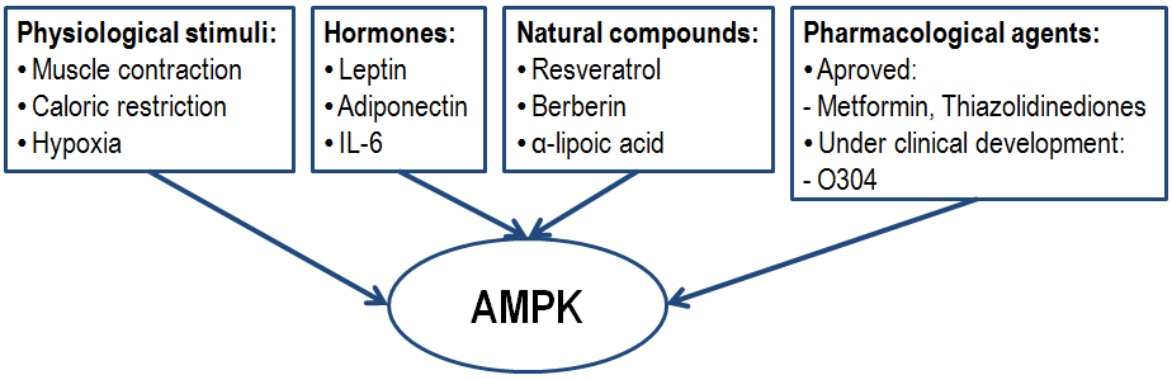


Figure 6: Different categories of AMPK's stimuli including physiological and pharmacological activators, hormones, and natural compounds. Most increase the AMP/ATP ratio and thus activate AMPK in an indirect manner. IL-6: interleukin-6, O304 – PAN-AMPK-activator, an anti-diabetic drug candidate in phase II clinical trials (Coughlan et al. [66])

Physiological ones are muscle contractions, hypoxia, and caloric restriction. They represent a rationale for physical exercise and strict diet in T2DM management. It has been demonstrated that AMPK reacts to the available cellular nutrients' levels, including glucose. In line with this, low glucose levels (3 mM or 54 mg/dL) activate AMPK through Thr172 phosphorylation, whereas high glucose levels (25 mM or 451 mg/dL) contribute to reduced AMPK phosphorylation and activity [69]. In addition, shear stress was also shown to activate AMPK in endothelial cells [70].

Hormones which can regulate AMPK are leptin, interleukin-6, and adiponectin [66]. Further details about the functions of the latter can be found under B-1.5.1. Certain natural compounds as the polyphenol resveratrol [71], and the alkaloid berberine [72] activate AMPK in an indirect manner by inhibiting mitochondrial ATP production, thus increasing the AMP/ATP ratio. In addition, α-lipoic acid used as a supplement or as a drug for the treatment of diabetes neuropathy in certain countries was also shown to activate AMPK [73]. Furthermore, many other approved anti-diabetic drugs exhibit additional beneficial effects through the same mechanism. For instance, it was conjectured that metformin activates AMPK indirectly through inhibition of the mitochondrial respiratory complex 1, similarly to berberine and resveratrol [19]. Metformin's main glucose lowering mechanism - inhibition of hepatic glucose production - is not likely to be AMPK-mediated, but it has been suggested that the kinase activation might contribute to increased insulin sensitivity [66].

2.2.2 AMPK – cellular functions and substrates

Along with the important functions of AMPK in organs, such as the liver and skeletal muscles, this kinase appears to have a key role in adipose tissue and in promoting processes that can be targeted by potential therapies of T2DM: AMPK increases browning of white adipose tissue, improves glucose uptake, and mitochondrial function of thermogenic brown adipose tissue resulting in elevated energy expenditure [74].

AMPK plays an important protective role for the cardiovascular system. It has been shown that activation of this kinase in endothelial and vascular smooth muscle cells contributes to vasodilation and reduces blood pressure [75]. AMPK is extensively investigated as a target for reduction of diabetes-related cardiovascular co-morbidities. A possible protective role can be explained with AMPK-mediated eNOS activation and release of NO [70].

Phosphorylation of downstream substrates is responsible for the catabolic effects of AMPK (Figure 7).

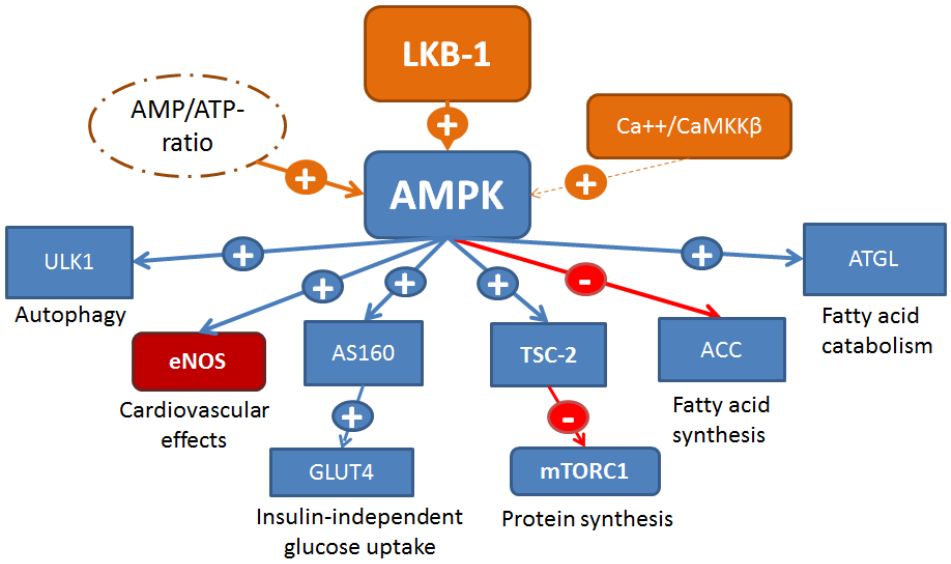


Figure 7: AMPK – the cellular metabolic switch. Increased adenosine monophosphate/adenosine triphosphate (AMP/ATP)-ratio, liver kinase B1 (LKB-1), and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) activate AMPK. Substrates: acetyl-CoA carboxylase (ACC) and adipose triglyceride lipase (ATGL) – responsible for fatty acids metabolism; 160 kDa Akt substrate (AS160) – mediating glucose uptake; endothelial nitric oxide synthase (eNOS) – responsible for cardiovascular effects; Unc-51 like autophagy activating kinase (ULK1) – promoting autophagy; Activated tuberous sclerosis complex 2 (TSC-2) inhibits mechanistic target of rapamycin complex 1 (mTORC1) and prevents protein synthesis.

Insulin-independent glucose uptake is mediated through AMPK, while insulin-dependent uptake is mediated through Akt. Each of these kinases manifest their activity by activating a pathway involving 160 kDa Akt substrate (AS160) and GLUT-4 [20]. AMPK inhibits acetyl-CoA carboxylase through phosphorylation which results in increased mitochondrial oxidation of free fatty acids [76]. Adipose triglyceride lipase (ATGL) is also regulated by AMPK [77]. Furthermore, AMPK activates tuberous sclerosis complex 2 (TSC-2) which, in turn, inhibits mTORC1 and prevents protein synthesis [78], but stimulates autophagy. The latter process is additionally promoted by Unc-51 like autophagy activating kinase (ULK1) [79].

In accord with the positive effects of AMPK-activation on numerous metabolic processes, a PAN-AMPK activator undergoing a phase II trial with metformin-treated T2DM patients remarkably improved their glucose homeostasis, microcirculation and blood pressure, and was considered as a successful candidate for a new anti-diabetic drug [77].

Most of the processes regulated by AMPK are involved in the pathogenesis of diabetes and metabolic disorders such as obesity or metabolic syndrome; ongoing research is presently investigating AMPK as a promising therapeutic target.

2.3 MAPK

MAPK (mitogen-activated protein kinases) is a family of protein kinases involved in the regulation of a variety of cellular processes such as proliferation, differentiation, survival, mitosis and stress-response. The conventional members of this kinase group are extracellular signal-regulated kinases 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). They can be activated in a receptor-dependent- or independent manner. Important stimuli are hyperglycemia, mediators of inflammation, and oxidative stress – factors present in T2DM and related metabolic disorders. The Ras/Raf/MAPK cascade plays an important role in the cardiovascular system and its dysregulation is related to cardiac hypertrophy, remodeling and atherosclerotic plaque formation (Figure 8) [80].

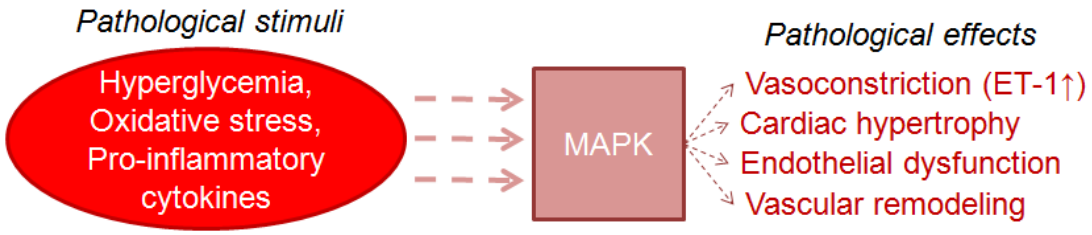


Figure 8: The role of mitogen-activated protein kinases (MAPKs) in the diabetes related cardiovascular morbidities. Hyperglycemia, oxidative stress, and the presence of pro-inflammatory mediators contribute to over-activation of MAPKs. In turn, they promote vasoconstriction (via endothelin-1; ET-1), endothelial dysfunction, cardiac hypertrophy, and vascular remodeling.

Sustained activation of ERK1/2, p38 MAPK, and JNK has been described as a hallmark of insulin resistance (A-1.4) and has been discussed as a substantial pathophysiological mechanism for diabetes-induced cardiovascular complications. Indeed, this was confirmed by a study indicating that altered signaling of MAPKs contributes to progressive development of endothelial dysfunction in a diabetic *in vivo* model [81]. That makes the MAPK signaling pathway an object of ongoing research.

3 Polyphenolic compounds

3.1 Background

Polyphenols are plant secondary metabolites and are prominently present in many human food sources, such as fruits, vegetables, nuts, spices and beverages [82]. This makes them important micronutrients in the human diet. In spite of their diversity these phytochemicals can typically be described as organic compounds with phenolic structural features in their molecules. More than 8000 structurally unique polyphenols with a plant origin have been identified so far [83]. In nature they most commonly occur as conjugates with sugar residues (i.e. D-glucose, L-rhamnose, D-galactose, L-arabinose, D-xylose) called glycosides.

Over the last 80 years polyphenols have been the subject of scientific interest because of their beneficial effects for different aspects of the human health [84]. Various epidemiological studies show the protective role of polyphenols against diseases, such as diabetes [85], cancer [86], cardiovascular [87], and neurodegenerative morbidities [88]. Ongoing research activities mainly engage in structure identification and quantitative analysis, pharmacokinetic characteristics and biological activities of polyphenols. Their spread use as food supplements demands strict guidelines and regulations for the quality and safety of these products [83]. Thus, interventional clinical studies with polyphenols are needed to verify their beneficial effects for humans, define optimal dosage regimens, and identify potential adverse effects.

3.2 Classification

The large number of already identified polyphenols and their structural diversity impose the use of a well-organized and descriptive classification. This is based on the number of phenol rings and the structural linkage between them. According to their scaffolds, polyphenols can be divided into the main subclasses of flavonoids, stilbenoids, lignans, and phenolic acids (Figure 9) [89-91]. An additional group including polyphenolic amides has also been suggested [92].

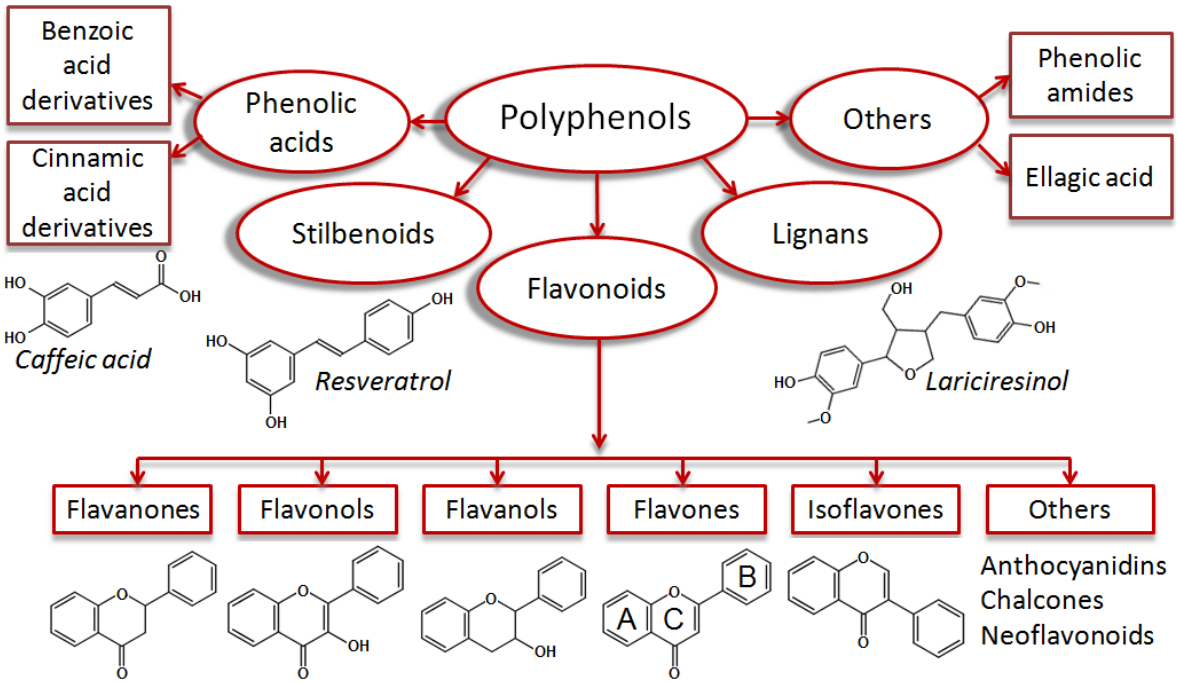


Figure 9: Classification of monomeric polyphenolic compounds according to Zhang et al., 2016 [89] with some modifications. Polyphenols are plant-derived substances composed of four main structural subclasses: phenolic acids, stilbenoids, flavonoids, and lignans. Phenolic acids are derivatives of benzoic or cinnamic acids (i.e. caffeic acid). Stilbenoids are hydroxylated stilbene derivatives (i.e. resveratrol). Lignans are diphenolic compounds (i.e. lariciresinol). Flavonoids are phenylchromone derivatives and in turn are divided into further subgroups: flavanones, flavonols, flavanols, flavones, isoflavones, and others (anthocyanidins, chalcones, and neoflavonoids). Some authors include phenolic amides (i.e. capsaicin) and ellagic acid derivatives [92] as further groups to polyphenols.

The group of flavonoids includes the highest number of representatives (around 4000) which are subdivided in further subclasses according to their structural characteristics. Monomeric flavonoids are low molecular weight substances with phenylbenzo-pyrone (phenylchromone) structure (Figure 9), based on a common three-ring nucleus. Two benzene rings (A and B) are linked through a heterocyclic pyrane ring (C) in between. Variations in the heterocyclic ring C such as the degree of saturation, the presence or the absence of carbonyl group (pyron/pyran), and hydroxyl groups, define the subclasses of flavones, flavonols, flavanones, flavanols, isoflavonoids, anthocyanidns, neoflavonoids and chalcones. A multitude of substitution patterns in the A and B rings gives a rise of various derivatives within each subclass of flavonoids. Typical substituents are hydroxyl and methoxy groups, O-glycosides, sulfates, and glucuronides [90]. Some of the most investigated flavonoids are quercetin, catechin, and genistein. Catechins can form dimers (e.g. procyanidin B1), oligomers, and polymers (proanthocyanidins). The latter are also called condensed or non-hydrolysable tannins. In contrast, polymerized glucose esters of

ellagic and gallic acids are known as hydrolysable tannins (ellagitannins and gallotannins) and are used as astringents [92].

Stilbenoids are hydroxylated derivatives of stilbene. Their scaffolds contain two phenyl rings connected by a two-carbon methylene linker. In contrast to flavonoids they are not that widely-spread and are often synthesized by plants in a response to abiotic stress. They can be found in grapes, red wine, peanuts and berries. Although a structural variety exists (i.e. oligostilbenoids), an object of scientific interest are mostly monomeric compounds, such as resveratrol and combretastatin A-4, because of their biological activities [93].

Lignans can be described as diphenolic compounds which are cinnamic acid derivatives. This group is characterized by a high structural diversity. Main dietary sources of lignans are flax and sesame seeds. After ingestion these polyphenols undergo extensive and rapid metabolism by intestinal microbiota resulting in compounds called enterolignans. Lignans act as phytoestrogens and possess some advantageous protective effects on cardiovascular system [94]. Typical representatives from this group are enterolactone, pinoresinol, and lariciresinol.

Phenolic acids are aromatic compounds having carboxyl functional group which are structurally relatively simple and can be divided into two subgroups: derivatives of benzoic acid and derivatives of cinnamic acid [90]. They are presented in many foods with plant origin, such as berries, grapes, nuts, black tee, and wheat. Extensively investigated representatives from this group are i.e. gallic, ferulic, and caffeic acid [91].

3.3 Beneficial effects

Epidemiological studies coupled and preclinical *in vitro* and *in vivo* investigations suggest that polyphenols exhibit plenty of health-promoting qualities. Their protective effects are considered pleiotropic, which means that they are not due to a single mechanism, but influence multiple signaling pathways and a variety of molecular targets. The most studied biological activities of polyphenols are related, but not limited to the following effects: anti-oxidant, cardio-protective, vasorelaxant, anti-thrombotic, anti-diabetic, anti-inflammatory, anti-proliferative, and neuroprotective. These activities are often not independent from each other, but might have overlapping underlying mechanisms. The mentioned effects in conjunction with low toxicity explain the polyphenols' role in prophylaxis and management of several diseases. The bioactivity of polyphenols in humans is determined by their pharmacokinetics. It is likely that metabolized forms of compounds available at the site of action are responsible for the observed effects rather than their chemically unchanged forms. This fact has often been disregarded.

3.3.1 Polyphenols and diabetes

Traditionally many medicinal plants were used for diabetes treatment. Polyphenols might be responsible, at least in part, for impacting different disease endpoints, such as glucose-lowering, insulin sensitizing, insulin secretion promoting, and inhibition of advanced glycation end products (AGE) formation. Polyphenolic compounds' protective effects on the β-cells have also been demonstrated [85]. Polyphenols exert benefits regarding late diabetes complications and therefore are comprehensively investigated as prophylactic tools and adjuvants for T2DM management (Figure 10).

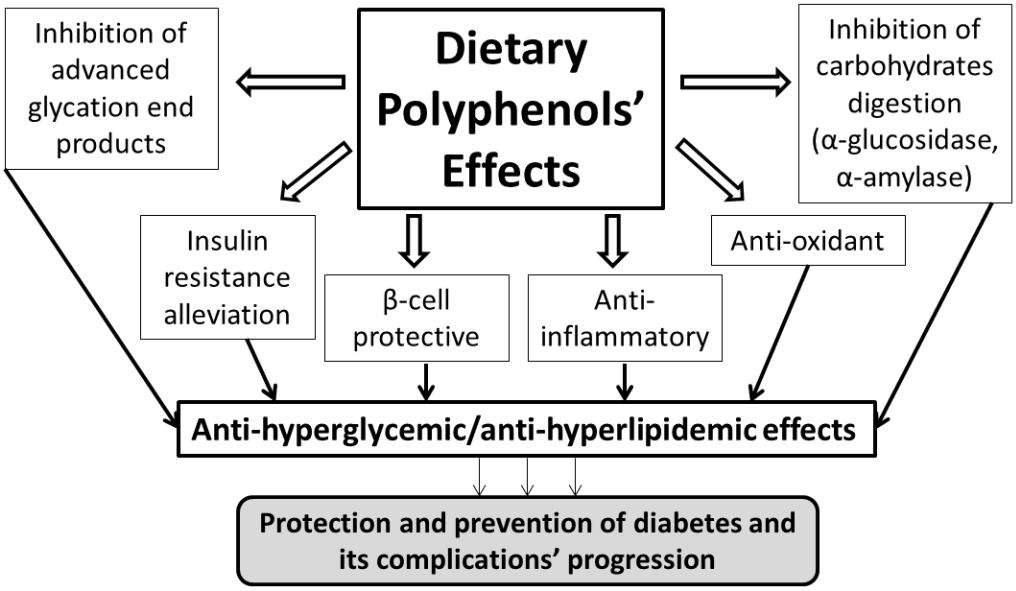


Figure10: Multiple aspects of antidiabetic properties of polyphenols (according to Xiao and Högger, 2014 [85] with some modifications).

Several epidemiological and interventional studies indicate that polyphenol-rich diet or intake of individual polyphenols can help preventing diabetes occurrence and to reduce its morbidity and mortality [95-97], although not all studies support this statement [98]. In addition, high polyphenol consumption influences obesity which is related to metabolic disorders and is a leading risk factor for insulin resistance and T2DM development, respectively [99]. Coffee is well-known as a source of dietary phenolics such as caffeic and chlorogenic acids. A systematic review with meta-analysis of 30 prospective studies showed that the coffee consumption is related with T2DM prevention and this effect appeared to be independent of caffeine [100]. As it has been suggested that dietary prevention of T2DM can be cost-effective approach, functional foods containing polyphenols may represent such an option [101].

The anti-hyperglycemic effect of polyphenolic compounds has been demonstrated in human studies. For instance, a randomized, placebo-controlled clinical investigation revealed that 12 week ingestion of apple polyphenols (600 mg/day) improved hyperglycemia after glucose challenge in the treated group of borderline subjects, while

placebo did not cause any effect [95]. Metformin in combination with a supplement called Emulin™ containing chlorogenic acid, myricetin, and quercetin showed superior anti-hyperglycemic effects compared to metformin alone in a placebo-controlled study with T2DM patients (n= 10 for each group). This suggests that certain polyphenols might be useful as an adjuvant anti-diabetic therapy [102].

The glucose-lowering effect of polyphenols is multifunctional, depending on the polyphenols' structure. Firstly, polyphenols may influence the carbohydrate digestion and the glucose absorption in the gastro-intestinal tract. Two key enzymes involved have been reported to be inhibited by polyphenols: α-amylase and α-glucosidase. As carbohydrates in food are the main source of blood glucose, inhibiting these enzymes is a promising strategy for blood glucose levels' regulation [103]. It has been reported that the length and complexity of polymer chain of tannins is favorable for α-amylase inhibition [85].

In addition, some polyphenolic compounds may improve an existing insulin resistance. A randomized, placebo-controlled, cross-over study showed that polyphenol-rich pomegranate juice consumption statistically significantly reduced fasting plasma insulin and alleviated insulin resistance (HOMA) in individuals with high cardiovascular risk [104]. A consumption of flavanol-rich chocolate statistically significantly decreased insulin resistance in impaired glucose tolerance patients, while flavanol-free chocolate had no effect (n= 19) [105]. However, chronic consumption of epigallocatechin gallate (EGCG, 400 mg/day) did not influence insulin resistance in overweight male subjects [106]. A basic research study pointed out that quercetin and resveratrol might mitigate insulin resistance in adipocytes *in vitro* through various mechanisms, such as attenuation of tumor necrosis factor-α- (TNF-α)-induced pro-inflammatory interleukins' gene expression, inhibition of MAPK-phosphorylation, promotion of peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling, and prevention of TNF-α-induced insulin receptor substrate 1 serine phosphorylation [107].

Advanced glycation end products (AGEs) play a major role in diabetes complications development and progression. They originate from non-enzymatic reactions between plasma proteins and reducing sugars. Thus, increased glucose levels in diabetes promote increased AGE-formation. After binding to respective receptors (RAGE), these covalent adducts activate pro-inflammatory processes, induce oxidative stress in endothelial cells [108], and reduce NO bioavailability [109]. Polyphenols might antagonize the negative effects of AGE on the cardiovascular system and thus delay late diabetes complications and disease progression. This protective effect can be achieved at different levels, such as scavenging of reactive oxygen species during glycation, inhibition of AGE-formation, blocking of RAGE-binding. Each polyphenolic subclass was reported to influence one or more of these processes [110].

3.3.2 Polyphenols and cardiovascular diseases

Cardiovascular diseases (CVD) are among the leading causes of death worldwide [91]. A strong relationship between the pathogenesis of T2DM and CVD has been established. Endothelial dysfunction, chronic pro-inflammatory state, insulin resistance, and hypertension are among the aspects defining cardiometabolic risk [111]. These factors favor atherosclerotic plaque formation and progression of CVD. A promising research field is polyphenols' effects on the cardiovascular system.

Various epidemiological studies showed an inverse association between polyphenol intake and occurrence of cardiovascular diseases [87, 112-114], including myocardial infarction [115, 116] and stroke [87, 117]. However, the mechanism of action of these nutraceuticals is complex and not fully clarified. The advantages of polyphenol-rich diet are demonstrated by so-called "French Paradox" – a lower incidence of cardiovascular mortality among the Mediterranean population in association with red wine consumption in spite of a diet high in saturated fats [118]. The latter is a well-known major risk factor for coronary disease [119]. In particular, red wine polyphenols as well as individual polyphenolic compounds found in red wine, such as quercetin, resveratrol, and catechin, attenuated the progression of atherosclerotic lesions *in vivo*, playing an important role in the development of several cardiovascular diseases [120]. Interestingly, quercetin's metabolite quercetin-3-O-glucuronide was shown to accumulate specifically in the foam cells in the injury site of blood vessels [121]. Ongoing clinical research focusses on the improved assessment of the impact of the polyphenols' intake on cardiovascular diseases [122, 123]. A current randomized, double-blinded trial investigates resveratrol's potential as an agent in heart failure management [124].

An interventional human randomized control parallel group study involving individuals with increased cardiovascular risk (n= 78) showed that consumption of polyphenols from different subclasses can promote cardiometabolic health [125]. The results revealed that the increase in flavone-intake had the strongest and statistically significant potential to improve post-challenge glucose response. In addition, postprandial lipid response was statistically significantly and inversely associated with intake of flavanones. Flavan-3-ols promoted early insulin secretion [125].

Many human studies attest that both individual polyphenols and polyphenol-rich foods exhibit mild anti-hypertensive effects [126-129]. A meta-analysis of ten randomized controlled clinical trials showed that flavanol-rich cocoa food exert slight blood pressure lowering effects in healthy and pre-hypertensive individuals. The mean change of the systolic blood pressure in the active treatment arms versus controls was –4.5 mmHg and for the diastolic one –2.5 mmHg. This reduction was statistically significant [126]. A double-blind, placebo-controlled cross-over study (n= 93) revealed that six weeks ingestion of the dietary polyphenol quercetin (150 mg/day) slightly, but statistically significantly decreased the systolic blood pressure (–2.6 mmHg) in treated individuals with

metabolic syndrome traits, when compared to the matched placebo arm [127]. However, the data for resveratrol's influence on the blood pressure in humans is controversial. A meta-analysis considering 17 randomized, controlled trials found that resveratrol had significant beneficial effects on hypertension only when applied in high doses (≥ 300 mg/day) and in diabetic patients [130].

Various flavonoids have been discussed to be capable of reducing blood pressure: flavonols, anthocyanidins, proanthocyanidins, flavones, flavanones, isoflavones and flavan-3-ols [131]. The improvement of flow mediated dilution (FMD, a non-invasive technique for measurement of endothelial function) caused by polyphenols was frequently reported [129]. This suggests that the amelioration of endothelial dysfunction by polyphenols is at least in part responsible for their beneficial effects on cardiovascular homeostasis, as demonstrated in animal models [132, 133].

Many *in vitro* and *in vivo* studies aimed to uncover the molecular mechanisms responsible for the mild vasorelaxant effect of these phytochemicals. An *in vitro* study with human aortic endothelial cells showed that polyphenols can potentiate insulin-triggered eNOS activation and NO release, causing a vasorelaxant effect [128]. Various studies also suggested Akt/eNOS pathway (see A-2.1.5) stimulation and NO production by polyphenols as a possible mechanism responsible for their vascular activity [134, 135]. Polyphenols were discussed to increase NO bioavailability due to their antioxidant effects [136]. Other possible targets of polyphenols investigated for their vasorelaxant effects include calcium channels in vascular smooth muscles [137], expression of the potent vasoconstrictor endothelin-1 [138], and inhibition of the angiotensin converting enzyme [139].

Polyphenols affect the inflammation involved in vascular diabetic complications and atherosclerotic lesions formation. It has been shown that polyphenols inhibit enzymes with key roles in inflammation, such as cyclooxigenase (COX), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS) [140, 141]. In addition, some polyphenolic compounds such as resveratrol reduce inflammatory mediators' levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and vascular cell adhesion molecule-1 (VCAM-1) [141]. On the cellular level this can be realized through interaction with pro-inflammatory pathways (p38 MAPK) and transcription factors [nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), nuclear transcription factor erythroid-2 related factor-2 (Nrf-2)] [142]. These activities add another aspect to cardiometabolic health promotion qualities of polyphenols.

Another pronounced activity of some polyphenols related to cardiovascular health is the antithrombotic one. In a pilot dietary intervention study with healthy individuals (n= 6), a single ingestion of 150 mg quercetin or 300 mg quercetin-4'-O-β-D-glucoside resulted in 4.66 µM, respectively 9.72 µM, peak plasma concentrations of quercetin after 30 minutes.

This intervention inhibited the platelet aggregation. The suggested mechanism involved inhibition of tyrosine phosphorylation within thrombocytes [143]. The use of flavonoids as templates for design of drugs with anti-thrombotic activity has also been discussed [144]. Further proposed mechanisms involved in anti-platelet effects of polyphenolic compounds are promotion of NO bioavailability, reactive oxygen species scavenging, cell-membrane binding, and antagonizing effects of pro-thrombotic mediators [144].

In addition, early research revealed that defined flavonoids ameliorated capillary fragility and tone – an action known as vitamin P-like effect. This property was used in a scurvy treatment [145]. Nowadays standardized polyphenolic extracts are available on the market for management of varicose veins and chronic venous insufficiency [146].

Pathological neovascularisation is a process described to occur in metabolic syndrome and leading to progression of diabetic nephropathy and retinopathy complications [147, 148]. It is responsible for unstable and leaky vessels. The usage of anti-angiogenic agents in the treatment of these complications has been proposed [148]. Polyphenols have been described to have anti-angiogenic properties and might be investigated in regard with this issue.

3.4 Safety of polyphenols

The wide use of polyphenols and their increased marketing as supplements raise the question about their potential harmful effects and safety profiles in general. In addition, presently no regulatory recommendations and no reference dietary doses for polyphenolic compounds have been suggested [149]. The consumption of polyphenol-rich fruits and vegetables is undoubtedly beneficial for the human health. However, intake of chemically pure substances or extracts should not be always expected to exhibit the same health effects or to be as harmless as the polyphenol-rich diet. Commonly, herbal dietary supplements are considered as safe which in some cases can be misleading and can result in wrong application or overdosage of these products [83]. Recently, it has been reported that approximately 20 % of medication-related liver injuries in U.S.A. were caused by food supplements, many of which containing polyphenols [150]. The shelf instability of certain compounds further complicates this issue. The main concerns regarding the consumption of polyphenols are related to potential carcinogenicity and mutagenicity, hepatotoxicity, nephrotoxicity, estrogen-like effects, and interactions with prescribed drugs.

Endeavors in preclinical and safety pharmacology research are aiming to assess potential toxicities of polyphenols and to reveal respective molecular mechanisms accountable for possible adverse effects. Among the most investigated substances are resveratrol, quercetin, catechins, and complex polyphenolic extracts.

Under certain conditions, such as excessive concentrations of phenolic antioxidants, presence of trace metal ions, and high pH-values, dietary polyphenols might exert harmful effects due to complex toxicodynamics. These compounds can easily get oxidized and thus can act as pro-oxidants, as demonstrated in *in vitro* and some *in vivo* studies [151, 152]. Resveratrol was shown to act as pro-oxidant *in vitro* causing the following negative effects: lipid peroxidation, DNA-damage, and mutagenesis [152]. Oxidized intermediates semiquinones can be involved in this process. Similar phenomenon was related to quercetin toxicity. "Quercetin paradox" denotes chemical conversion of the flavonol into oxidized products after scavenging of free radicals, i.e. ortho-quinone or quinone methide intermediates [153]. These species are highly reactive and can modulate proteins causing loss of their functions. Thus, initially quercetin is capable of protecting cells from oxidative stress, but the following formation of its toxic oxidized products can lead to glutathione depletion, covalent protein binding, lactate dehydrogenase leakage and cell dead [153]. Therefore, although seemingly controversial, polyphenols can act either as anti- or pro-oxidants, depending on the cellular environment and applied concentrations. Polyphenols can act as antioxidants at low concentrations, while high concentrations can be potential prerequisite for toxicities [151, 152]. Similar pro-oxidative activities *in vitro* have been described for flavan-3-ols as well [151]. Considering the results from *in vitro* studies, investigations aiming to establish if these findings are valid *in vivo* have been performed [152, 154]. Outcomes from animal studies revealed maximal tolerated doses for some polyphenols in rat models by oral administration. A two years long intake of quercetin at a dose of 500 mg/kg body weight (b.w.)/day was considered safe [154]. No adverse effects following 28 day resveratrol consumption (1000 mg/kg b.w./day) were observed [152]. Nephrotoxic effects for both polyphenols were registered after higher dose intake (>2000 mg/kg b.w./day). However such doses are corresponding to a daily intake of over 140 g pure substance and are not expected to be ingested by humans.

In addition, certain polyphenolic subclasses act as phytoestrogens due to their structural features [152]. These effects are most pronounced for isoflavones, stilbenoids and lignans [155]. Phytoestrogens can act as either agonist or antagonist of estrogen receptors and thus can influence key cellular processes. This raises some safety concerns related to the embryonal development, estrogen-sensitive cancer occurrence and growth, and gynecomastia [83]. Based on recent epidemiological studies, the European Food Safety Authority stated that the intake of isoflavone-containing supplements is secure for peri- and post-menopausal women [156]. However, possible issues related to estrogen-like effects of polyphenols should not be disregarded.

A growing body of evidence gained by case studies and interventional clinical trials for consumption of polyphenols contributes to critical evaluation of their safety profiles in humans. A comprehensive literature review described 19 cases of hepatotoxicity related to the catechin-rich green tea supplementation with different severity of side effects [157]. It had been suggested that patient susceptibility and liver disease history might contribute to

negative events, but the mechanism of the hepatic injuries remains largely unclear. A more recent report of the European Food Safety Authority also addressed the issue for green tea catechins [158]. The consumption of green tea supplements was considered safe, and the rare cases of liver injury were suggested to be due to idiosyncratic type adverse reactions. Importantly, the intake of over 800 mg/day EGCG was related to statistically significant increase of serum transaminase activities [158]. Thus, green tea supplements should be preferably taken after careful risk-benefit assessment and under medical surveillance, especially for patients with previous liver injuries.

Clinical studies performed with quercetin gave insights for its safety [127, 143]. In a double-blind, placebo-controlled cross-over study the ingestion of 150 mg/day of the flavanol for six weeks was explored. The biochemical and hematology parameters investigated as secondary outcomes did not show any side effects of quercetin on the liver and kidneys [127]. In a phase I clinical trial with quercetin Ferry et al. studied the maximal tolerated doses of the parenterally applied flavonoid through *i.v.* bolus either weekly or in a three-week interval in cancer patients (n= 51) [159]. At a low dose (~10 mg/kg b.w.) only mild adverse effects were observed, e.g. moderate local pain, flushing and sweating. When ~50 mg/kg b.w. quercetin was applied, dose-limiting toxicities included: acute renal failure, clinically significant nephrotoxicity and renal impairment. In addition, dyspnea, nausea and vomiting, but not myelosuppression have been reported [159].

Likewise, resveratrol was tested in clinical studies which revealed its low toxicity in humans [130, 152]. Doses of 5 g/day for up to one month were described as safe and well-tolerated in healthy individuals [152]. A randomized, double-blind, placebo-controlled trial of the effects of resveratrol at a dose up to 1000 mg twice daily for 52 weeks in moderate Alzheimer disease patients revealed some mild adverse events. The most common ones were nausea, diarrhea, and weight loss [160].

Another potential risk related to the intake of polyphenols might be their interactions with some drug-metabolizing systems or drug transporters. This can decrease or increase bioavailability of drugs and possibly result in serum concentrations outside their therapeutic windows. Indeed, quercetin was reported to inhibit cytochrome P-450 CYP2A6 and P-glycoprotein *in vivo* which potentially can cause drug interactions [161]. Resveratrol has been described to inhibit CYP3A4 *in vivo* and *in vitro* [152]. A potential interaction between quercetin and tamsulosin on the pharmacodynamic level resulting in increased vasorelaxation has been suggested based on the results from an *ex vivo* model employing rat mesenteric arteries [162]. Therefore, administration of polyphenols should always be checked for compatibility with assigned therapies.

Another general concern is related to unspecific molecular effects of phenolic compounds. Polyphenols have been described to cause perturbations of membrane phospholipid bilayers and in this way to modify certain membrane protein functions in a promiscuous

manner [163]. Such effects were registered for resveratrol and genistein at concentrations higher than 10 µM [163]. In addition, some representatives (e.g. quercetin) have been shown to be large panel kinase inhibitors [164]. Thus, in contrast to many approved drugs designed to affect selectively and specifically a define target within the body, polyphenols are capable of influencing multiple signaling pathways and expression of different genes simultaneously. This particularity should be taken into consideration in approaches aiming at the increased bioavailability of polyphenols over the dietary consumption, i.e. use of prodrugs or drug delivery systems.

In summary, polyphenols are unlikely to cause severe adverse events in humans in case of proper preparation, storage, and consumption.

3.5 Pycnogenol®

Pycnogenol® (PYC) is a standardized extract of French maritime pine bark which contains 65-70 % oligomeric procyanidins, polyphenolic monomers, and phenolic acids [165]. It complies with a monography of the United States Pharmacopeia (USP) and is widely marketed as a food supplement. Monomeric compounds include bioflavonoids catechin and taxifolin, and phenolic acids, such as caffeic, ferulic, and cinnamic ones, which are present as both aglycones and glycosides. Inorganic ions, such as calcium, potassium and iron are presented in the extract as well [166].

3.5.1 Pycnogenol® – beneficial effects

PYC consumption has been shown to promote cardiometabolic health in humans. Various clinical studies indicate beneficial actions of this extract in patients with T2DM and metabolic syndrome, especially: anti-hyperglycemic, glycosylated hemoglobin-lowering [167, 168], and α-glucosidase inhibiting effects [169]. Human studies showed that PYC can improve diabetic late complications such as microangiopathy [170] and rethinopathy [171, 172]. Additionally, PYC was shown to beneficially influence T2DM-related metabolic disorder parameters such as blood pressure, total cholesterol, fasting glucose [173], triglyceride levels and HDL (high density lipoprotein) [174] which had also been confirmed in clinical trials. Recent studies demonstrated that PYC can prevent recurrent venous thrombosis and post-thrombotic syndrome, and can successfully be used in the management of chronic venous insufficiency [146, 175].

A recent review summarized additional advantageous effects of PYC regarding aging-related diseases, such as osteoarthritis, cognitive dysfunctions, climacteric symptoms, etc. [176].

3.5.2 Pycnogenol® – pharmacokinetics

The question how exactly PYC works is not completely clarified and its pharmacokinetics are important aspect of the answer. A clinical study involving healthy volunteers aimed to uncover the presence of polyphenols and their metabolites in human plasma resulting from PYC consumption [165]. After ingestion, some of the monomeric extract ingredients got rapidly absorbed. Catechin, ferulic acid, and caffeic acid were detected in plasma even 30 minutes after a single PYC intake (dose 300 mg). Oligomeric compounds got gradually metabolized pre-systematically by intestinal microbiota providing sustained release of catechin monomers. Therefore, the maximal plasma concentration of catechin was detected after four hours and relatively constant plasma concentrations were presented up to 14 hours. Intestinal microbiota converts catechin into its metabolite M1, which is detected in plasma after PYC ingestion, but is not originally presented in the extract. Accordingly, M1 appeared in plasma at first after six hours and its peak plasma concentrations were achieved after ten hours. Taxifolin's maximum concentrations were shown after eight hours. In addition, ten unknown compounds have been detected [165]. Some of them probably resulted from intestinal microbiota metabolism. This study gave a hint for the absorption of PYC's ingredients and steady state concentrations of original constituents and their metabolites. A randomized controlled interventional clinical trial involving osteoarthritis patients (n= 33) investigated the distribution of PYC's ingredients and metabolites into different body compartments after a three week intake of 200 mg/day standardized French maritime pine bark extract [177]. The results showed high interindividual variations in the measured serum concentrations of PYC's ingredients and metabolites among the participants. These compounds were detected in the nanomolar concentration range and their degree of conjugation with glucuronic acid and sulfate was variable: from ~50 up to 98 %. Some compounds were preferably distributed in blood cells or in the synovial fluid rather than in serum. The concentrations of bioflavonoids catechin and taxifolin were higher in the blood cells than those in serum. Ferulic and caffeic acids and the metabolite M1 were detected in the synovial fluid in higher concentrations than in serum. Thus, it was demonstrated that polyphenols predominantly accumulated at the site of injury despite their low serum concentrations. PYC was well tolerated in this study and no adverse events were registered [177]. Ferulic acid, taxifolin, and M1 were shown to be excreted in the urine [178]. Identification of the unknown PYC metabolites is of interest, as some of them can be responsible for the beneficial effects of the extract described in many clinical studies (A-3.5.1).

3.5.3 Pycnogenol® – pharmacodynamics

Only *in vitro* experiments employing relevant polyphenolic compounds at physiologically relevant concentrations, as opposed to using the whole polyphenolic extract, can be considered useful to provide a hint for the pharmacodynamics of PYC responsible for its health promoting activities.

Without a doubt, the antioxidant properties of PYC's ingredients contribute to its health benefits and protective effects. A placebo-controlled study revealed that the intake of 50 mg PYC daily over eight weeks reduced statistically significantly reactive oxygen species *in vivo* in healthy smokers (n= 78) in comparison to the matched control group (n= 77) [179]. This outcome approved results from *in vitro* study showing antioxidant potential of PYC's ingredients and metabolites [180]. In addition, the French maritime pine bark extract has been shown to modulate some enzymes with important role in the inflammatory response. An *ex vivo* study with serum obtained from volunteers 30 min after treatment with PYC showed inhibition of cyclooxigenases (COX-1 and COX-2), influencing prostaglandin-generation inflammatory pathway [181]. Likewise, similar *ex vivo* approach showed that human plasma probes after PYC ingestion inhibited NF-κB signaling pathway and decreased metalloproteinase-9 secretion by human monocytes. These studies suggest that bioavailable and bioactive compounds associated with the PYC's intake are responsible for the documented anti-inflammatory effects of PYC in humans. Additional metalloproteinases and iNOS are possible targets of the extract's active principles [140, 180]. An extensive review further discussed pharmacodynamical mechanisms and implications of PYC's antioxidant and anti-inflammatory effects in the management of metabolic syndrome and its related disorders [182].

4 Aims of the thesis

The aim of the present work was the characterization of cellular effects of variety of individual polyphenols and of polyphenol rich extract PYC, which may have importance in the prevention and management of T2DM and its late complications.

The effects of PYC on diabetes relevant targets, such as intact glucagon-like peptide-1, dipeptidyl peptidase IV (DPP IV), relaxin-2, and adiponectin were to be investigated in an interventional clinical trial (B-1.1).

The elucidation of the *in vitro* activities of individual ingredients of PYC and their metabolites on DPP IV were of interest and aimed to characterize more accurately the polyphenol's effects at the molecular level. This investigation involved two aspects: Firstly, the inhibitory potentials of polyphenolics on the enzymatic activity of DPP IV were to be analyzed (B-3.1). Secondly, the impact of these compounds on the total protein expression of DPP IV *in vitro* was to be elucidated (B-3.2).

Akt-kinase plays an essential role in T2DM and its vascular long term complications (as already described under A-2.1). Therefore, how polyphenols influence the phosphorylation of Akt in endothelial cells was of interest. It was aimed to develop and validate an assay for investigation of the phosphorylation status of Akt-kinase (B-2.1). For this purpose, quercetin and resveratrol were used as model compounds, as evidence suggested them as beneficial for cardiovascular health [183-185] and abundant ingredients in the human diet [186].

A following study (B-2.2) aimed to investigate the effects of bigger group of polyphenols from different structural subclasses on the phosphorylation of Akt at Ser473 and Thr308. Afterwards, in a comprehensive screening (B-2.3) a detailed investigation of numerous polyphenols for their effects on the phosphorylation of Akt at Ser473 in endothelial cells by high-throughput and quantitative method (C-2.4.5) was to be performed. Thereby a basis of subsequent structure-activity-relationship studies (SAR) was to be created. Possible effect of bio-activation through bio-transformation was studied as a part of the screening. Thus, the *in vitro* activities of precursor substances on endothelial Akt-phosphorylation were compared with those of their intestinal microbiota generated metabolites (B-2.6).

As Akt is a target with relevance to T2DM, it was investigated whether certain polyphenols have a differential effect on the Akt-phosphorylation under physiological and hyperglycemic conditions (B-2.8). In addition, the effects of polyphenols were compared with those of the anti-diabetic drug metformin to determine if these compounds might possess "metformin-like" activities on endothelial cells mediated through Akt-inhibition (B-2.7).

B Results and Discussion

1 Cellular effects of Pycnogenol® in healthy individuals

As PYC possesses glucose-lowering effects in diabetics (A-3.5.1) it was of interest in relation to its safety whether the extract can influence blood glucose levels and some diabetes related target molecules in healthy individuals. In order to clarify the role of the described α-glucosidase inhibition for PYC's effects on glucose homeostasis, a comparison between responses of healthy participants to oral glucose tolerance test with glucose and one with boiled rice as a source of complex carbohydrates was performed.

1.1 Pycnogenol® effects on active GLP-1, DPP IV, relaxin-2, and adiponectin

The impact of PYC on intact GLP-1 (glucagon-like peptide-1), DPP IV (dipeptidyl peptidase IV), relaxin-2 and total adiponectin serum concentrations, as well as on DPP IV serum enzymatic activities was investigated in serum samples obtained in a clinical study performed at the cooperating Comenius University, Bratislava. Twenty (n= 20, ten male and ten female) healthy participants were included into this interventional clinical study (Figure 11). The study consisted of a run-in period (14 days) and an intervention period (14 days). During the intervention period PYC was taken at a dose of 100 mg/day. On the days of oral glucose tolerance test (OGTT), PYC was administered 30 minutes before the start of the carbohydrate challenge. OGTT-1-G was the first OGTT performed with 75 g anhydrous glucose *per os* at time point T1 at the end of the run-in period. Likewise, OGTT-1-R was the first oral glucose tolerance test performed with 200 g boiled rice, corresponding to 50 g of glucose at time point T2, which was also at the end of the run-in phase. OGTT-2-G was the second oral glucose tolerance test performed with glucose at time point T3 after the intervention period. Accordingly, OGTT-2-R was the second oral glucose tolerance test performed with rice at time point T4.

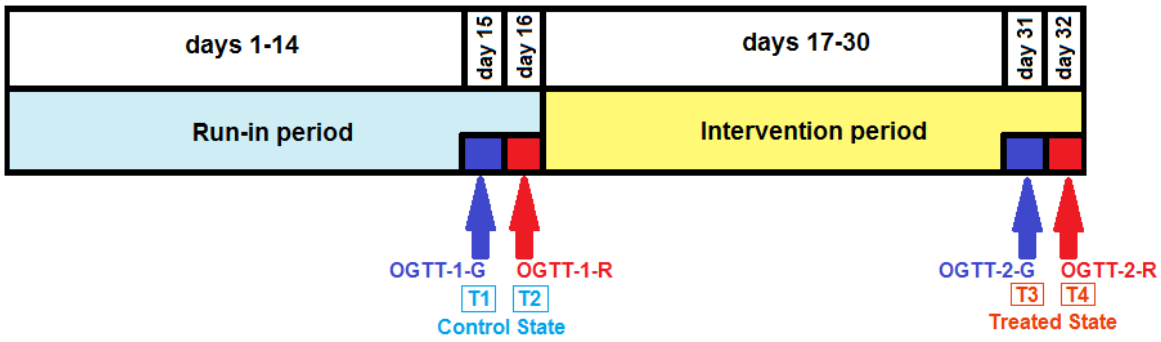


Figure 11: Protocol of the clinical study. The run-in period was 14 days. T1 – time point of OGTT-1-G (first oral glucose tolerance test performed with 75 g glucose *per os*) before Pycnogenol® supplementation. T2 – time point of OGTT-1-R (first oral glucose tolerance test performed with 200 g boiled rice) before Pycnogenol® supplementation. The intervention period lasted 14 days. T3 – time point of OGTT-2-G (second oral glucose tolerance test performed with 75 g anhydrous glucose *per os*) after 2 weeks of Pycnogenol® supplementation. T4 – time point of OGTT-2-R (second oral glucose tolerance test performed with 200 g boiled rice) after 2 weeks of Pycnogenol® supplementation.

Thus, the study proceeded in four phases:

- T1 – control state: after two weeks run-in period, before Pycnogenol® (PYC)-intake, classic OGTT with glucose.
- T2 – control state: after two weeks run-in period, before PYC-intake, boiled rice equivalent OGTT.
- T3 – treated state: after intervention period of two weeks PYC-intake, classic OGTT.
- T4 – treated state: after intervention period of two weeks PYC-intake, boiled rice equivalent OGTT.

The volunteers' samples were coded as following: X-TY-Z, with X being the number of the participant (1-20), TY (Y = 1, 2, 3 or 4) indicating the phase of the study, and Z (Z = 0, 30, 60, 90, 120) describing the time (in minutes) of the blood collection during the OGTT.

1.2 Effects of Pycnogenol® on GLP-1 (active)

1.2.1 Background: GLP-1 (active) serum levels

Incretin hormones are released from gastro-intestinal tract cells after meal ingestion and promote secretion of insulin from pancreatic β-cells, outlining the enteroinsular axis. The best-studied incretin is glucagon-like peptide-1 (GLP-1), produced by ileum and colon L-

cells [187]. Its physiological actions such as postprandial insulinotropic effect, advancement of β-cells growth and survival, inhibition of glucagon release, and promotion of satiety feeling are mediated by G-protein coupled receptors [188]. These functions explain its important role in the postprandial glucose homeostasis. The potential of GLP-1 and its receptor ligands to improve diabetes-related endothelial dysfunction [189] and their role as cardioprotective agents [190] have also been discussed. GLP-1 is firstly produced by processing glucagon as biologically inactive precursor forms GLP-1(1-37) and GLP-1(1-36) amide (intestinal forms). Afterwards, two active (indicated here as active/intact GLP-1) equipotent forms are generated by cleavage of six N-terminal amino acids: glycine-extended one - GLP-1(7-37) and amide one - GLP-1(7-36a). The first one is predominant in circulation. GLP-1 is subjected to proteolytic degradation by dipeptidyl peptidase IV (DPP IV). Thus, the half-life of the active GLP-1 is very short (ca. 2 min), it is rapidly transformed into the inactive GLP-1 metabolites (GLP-1 (9-37) and GLP-1 (9-36a)) (Figure 12) [187].

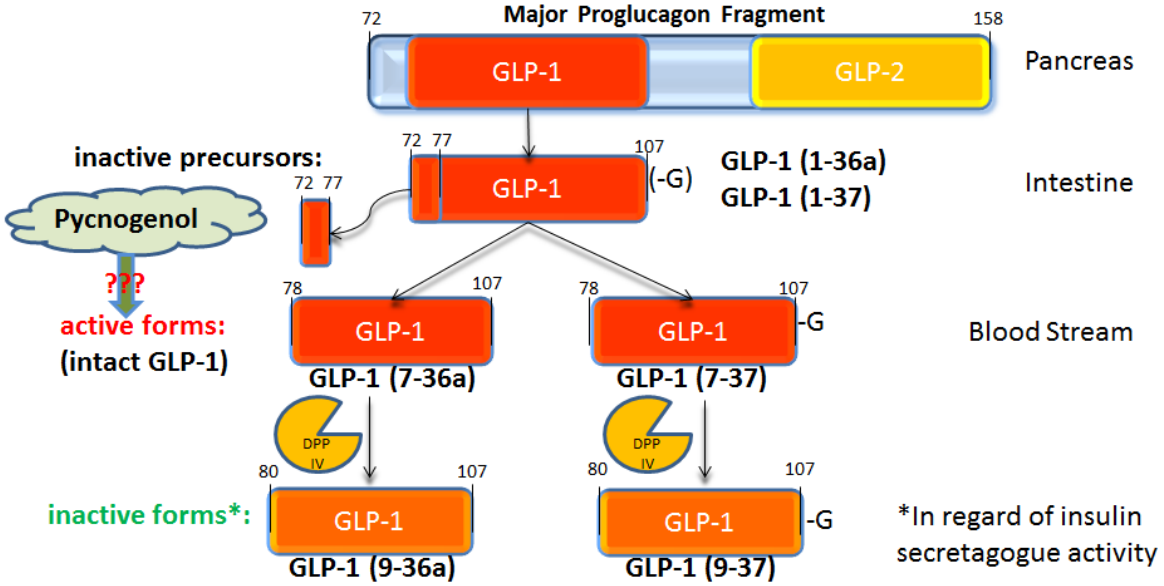


Figure 12: Glucagon-like peptide 1 (GLP-1) forms. Two active/intact GLP-1 forms are known, namely GLP-1 (7-36a) and GLP-1 (7-37). The active forms are subjected to proteolytic degradation by DPP IV (dipeptidyl peptidase IV) which results in loss of insulin secretagogue activity. The numbers indicated on the fragments represent the amino acid sequence of the precursor protein (major proglucagon fragment). The present study aimed to investigate whether Pycnogenol (PYC) possesses any effects on the serum levels of intact GLP-1 in healthy subjects.

1.2.2 Results: Effects of Pycnogenol® on GLP-1 (active) serum concentrations

1.2.2.1 Classic OGTT protocol

Both before and after the intervention period, the glucose charge caused an increase in the intact GLP-1 levels, compared to the fasting state: T1-30 mean (9.98 ± 2.80 pM) > T1-0 mean (8.93 ± 2.97 pM) and T3-30 mean (9.95 ± 3.34 pM) > T3-0 mean (8.70 ± 3.23 pM). However, these differences were not statistically significant (p= 0.45, one-way ANOVA). The basal mean concentration of intact GLP-1 was slightly (but not statistically significantly) decreased by 0.23 pM after two weeks Pycnogenol-intake period (Figure 13.1).

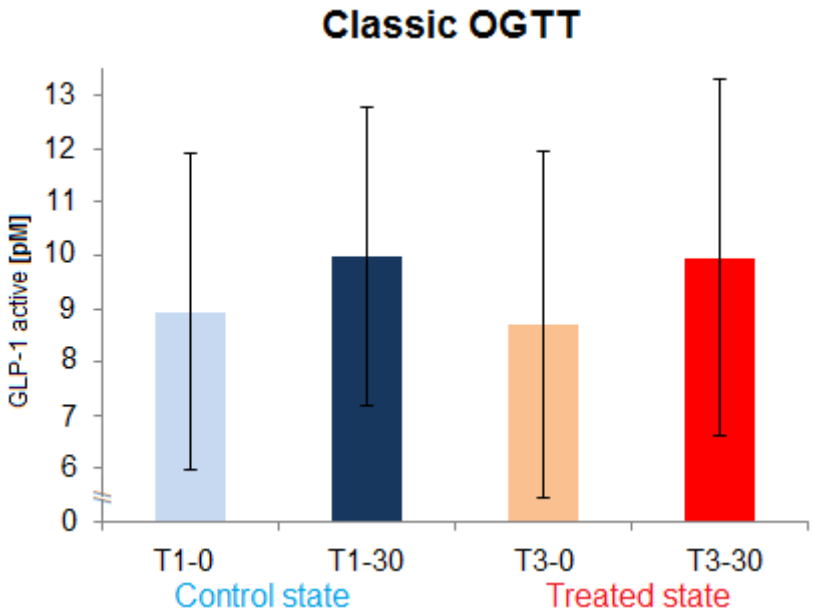


Figure 13.1: Serum concentrations of GLP-1 (active) in healthy individuals before (T1-0, T1-30) and after 2 weeks of PYC supplementation (100 mg/day) (T3-0, T3-30). Columns represent means and standard deviation. Classic OGTT caused slight, but not statistically significant increase in GLP-1 (active) levels, compared to the fasting levels. No significant changes in GLP-1 (active) levels were induced by PYC (p= 0.45, n= 19).

The mean values, standard deviations (S.D.), medians, mean deviation (Mean Dev.) are indicated in the Supplement, Section D-1.1).

Four values (8-T1-0, 8-T1-30, 8-T3-0, 8-T3-30) were identified as outliers (as described in C-1.5.3) and were excluded from further data analysis. The values of all measured individual concentrations can be found as a supplementary attachment (Supplement, Section D-1.1).

1.2.2.2 Boiled rice equivalent OGTT protocol

The boiled rice administration caused non-significant rise in the intact GLP-1 concentrations, compared to the fasting state, both before (T2-30 mean (10.19 ± 4.33 pM) > T2-0 mean (8.87 ± 4.42 pM)) and after Pycnogenol®-intake period (T4-30 mean (9.24 ± 4.82 pM) > T4-0 mean (8.27 ± 3.43 pM)). Additionally, the difference between fasting mean levels and boiled rice-induced GLP-1 mean levels was slightly smaller, after the PYC intervention period (treated state) [mean (T4-30) – mean (T4-0) = 0.97 pM] compared to the control state [mean (T2-30) – mean (T2-0) = 1.32 pM]. Similarly to the results obtained after the classic OGTT protocol, the mean baseline concentration of intact GLP-1 was slightly lower (with 0.6 pM) after the intervention period (T4-0) in comparison to that one before (T2-0), (T2-0, T2-30, T4-0 – n= 19, T4-30 – n= 20). Nevertheless, none of the mentioned differences were statistically significant (p= 0.57, one-way ANOVA) (Figure 13.2). Three values (8-T2-0, 8-T2-30, 8-T4-0) were identified as outliers (C-1.5.3).

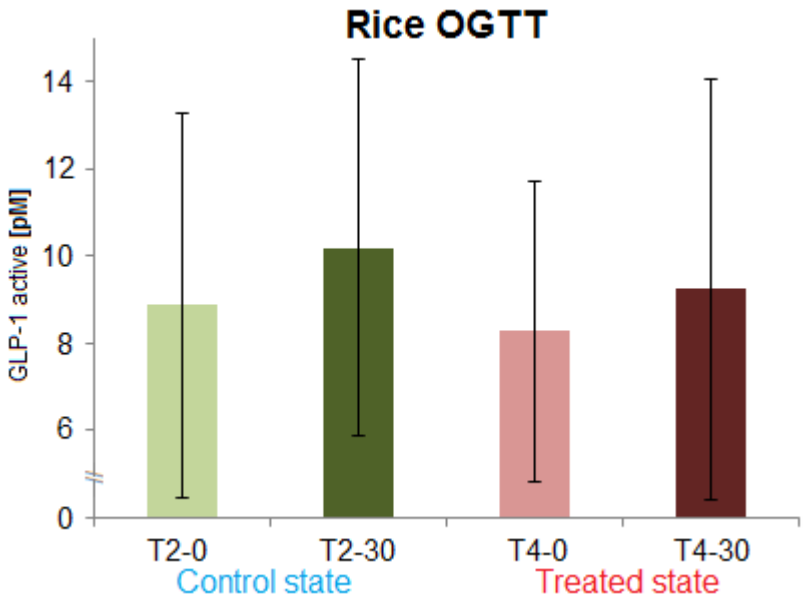


Figure 13.2: Serum concentrations of GLP-1 (active) in healthy individuals before (T2-0, T2-30) and after 2 weeks PYC intake (100 mg/day) (T4-0, T4-30). Columns represent means and standard deviation. Rice OGTT caused slight, but not statistically significant increase in GLP-1 (active) levels, compared to the fasting levels. No statistically significant changes in GLP-1 (active) levels were induced by PYC (p= 0.57, for T2-0, T2-30, T4-0 –n= 19, for T4-30 – n= 20).

Means, standard deviations, medians, and mean deviations are indicated in Supplement, Section D-1.1.

1.2.2.3 Individual Changes in GLP-1 (active) serum concentrations before and after Pycnogenol® intake

Individual changes in active GLP-1 levels for each participant between states T1-T3 and T2-T4 were calculated. As it was no difference in the conditions before the performance of OGTT (or rice OGTT) at time point 0' the data before PYC-intake (T1-0, T2-0) were combined as a control state and the phases after PYC-intake (T3-0, T4-0) were combined as a treated state. The absolute values were normalized to the mean of the control state (100 %), (Figure 13.3).

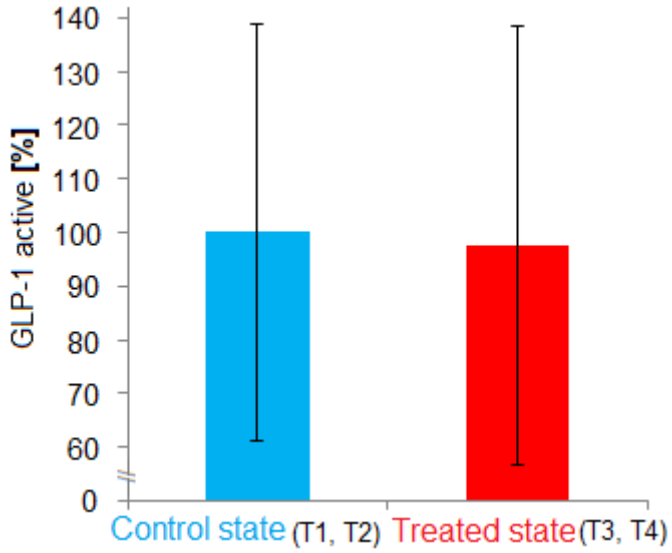


Figure 13.3: Individual changes of GLP-1 (active) levels (at time point 0') in healthy subjects before and after 2 weeks PYC-intake (100 mg/day). Data were normalized to the values before PYC-intake and before OGTT (T1-0, T2-0). The mean of individual changes indicated a minor and non-statistically significant decrease in fasting GLP-1 (active) levels of 2.55 % after PYC-intake compared to the control state (p= 0.71, two tailed paired Student's t-test, n= 35).

The mean of the individual changes of all of the samples included into the analysis indicated a slight decrease of 2.55 % in the intact GLP-1 concentrations after PYC intake (at 0'), compared to the control state. This change was not statistically significant (p= 0.71, two tailed paired Student's t-test, n= 35). Five values (absolute values: 8-T1-0, 8-T3-0, 8-T2-0, 8-T4-0, and normalized ratios: respectively 8-T1-0/T3-0, 8-T2-0/T4-0, and 10-T2-0/T4-0, 11-T1-0/T3-0, 14-T2-0/T4-0) were identified as outliers by Grubbs' test and box plot tests [191, 192] and were excluded from the analyzed data. (The values of individual change for each participant are shown in the Supplement, Section D-1.1).

1.2.3 Discussion: Effects of Pycnogenol® on GLP-1 (active) serum levels

The data from the present study showed an expected tendency for rapid increase in intact GLP-1 levels upon OGTT performed with glucose or a boiled rice equivalent. Probably due to the small sample size and the high data variability this tendency was not statistically significant. The increases in GLP-1 concentrations are characteristic for previously described meal-triggered GLP-1 postprandial response [193]. Generally, an increase of GLP-1 concentrations in response to a glucose challenge was expected as oral nutrients such as glucose and fat have been proven to be rapid and potent triggers of GLP-1 secretion [194]. The mean concentrations of active GLP-1 measured in the present study (8.27 – 10.19 pM) were comparable to the concentrations reported for healthy participants in other studies. Ryskjær et al. (2006) and Vilsboll et al. (2001) reported similar fasting levels of intact GLP-1 of 9.0 ± 3.5 pM [195] and 9.0 ± 1 pM, respectively [196]. Slightly lower intact GLP-1 concentrations were published as well: 5.2 pM (median with an interquartile range 3.1 – 8.4 pM) [197]. The present data showed that the levels of GLP-1 (active) were not elevated in healthy participants after consumption of PYC compared to the control state, and thus a potential GLP-1-mediated hypoglycemia [198] should not be expected. However, in prediabetes and T2DM patients a different response to PYC-treatment might be observed. In the present study GLP-1 (active) serum levels in healthy individuals tended to increase after 30 minutes minimally by 11.7 % and maximally by 16.06 % as a response to OGTTs in all trial phases (B-1.2.2.1and B-1.2.2.2). The outcome of a longitudinal risk-stratified cohort study including 525 personals with prediabetes and 774 healthy controls suggested that GLP-1 secretion response to OGTT is reduced in prediabetes: 18-25 % lower in women with prediabetes, compared with normal glucose tolerance (NGT) women, and 16 % lower in men with prediabetes, compared with NGT men [199]. Another human study investigating male patients with obesity and T2DM (n= 10) revealed no significant variation in intact GLP-1 forms after glucose challenge as opposed to obese, but non-diabetic controls (n= 20) [200]. This suggests significant differences in serum GLP-1 levels between healthy volunteers, prediabetic and diabetic patients and probability that the GLP-1 secretion response to glucose is related to the stage and progression of the disease. PYC can be considered as safe in healthy humans regarding postprandial reactive hyperglycemia risk. Its glucose-lowering action in T2DM patients is further to be investigated.

There was no significant difference between the mean fasting intact GLP-1 concentrations before (T1-0 and T2-0) and after the 2-weeks PYC-intake period (T3-0 and T4-0). This result is consistent with the results for insulin and glucose levels from the same study (data from Prof. Ďuračková) with no significant increases in the insulin levels after 30 minutes upon OGTT being observed between control and treated state after 2 weeks PYC-intake. This is consistent with the study of Liu X et al. [168] suggesting that the glucose-lowering effect of PYC in T2DM patients probably is not due to enhanced insulin secretion.

A minor and non-significant decrease (–0.23 pM and –0.6 pM for T1/T3 and T2/T4, respectively) was observed in the mean GLP-1 (active) levels after PYC-intake intervention period in comparison to the control state before PYC-intake in the investigated volunteer group. This difference was probably due to large interindividual variations.

The slightly smaller increase of intact GLP-1 in boiled rice-equivalent protocol after PYC-intake (T4 phase) in response to OGTT in comparison to all other phases might be due to α-glucosidase inhibitory effect of PYC [169]. Such a reduction of GLP-1 response was not observed after PYC-intake in classic OGTT protocol performed with glucose. Thereby, the release of glucose from rice starch might be decreased and therefore the secretion of GLP-1 could be reduced or delayed. However, the glucose time course from this study (data from Prof. Ďuračková) does not support such an α-glucosidase inhibitory effect. A likely explanation is that α-glucosidase inhibitors (such as acarbose, voglibose) should be taken simultaneously with the meal in order to achieve a maximal effect [201]. In contrast, PYC was taken 30 minutes before the OGTTs, which might contribute to a different outcome.

The available data on effects of polyphenols on intact GLP-1 levels in humans is generally sparse. Berries considered as a rich source of polyphenols were investigated in a small human study with 12 healthy volunteers (randomized, placebo-controlled, crossover) who took part in two single meal tests on different days. The outcome showed modest enhancement on the GLP-1 response to sucrose, which was not statistically significant [193]. Another small study (double blind, placebo-controlled, crossover), with 8 T2DM individuals investigated the acute effects of standardized bilberry extract and reported a significant decrease in blood glucose concentrations, but no change in GLP-1 [202]. A study with 9 healthy participants (randomized, crossover) investigated the effects of a single intake of beverages containing chlorogenic acid on GLP-1 secretion and found no significant increase after 30 minutes compared to the controls. In this study it was not specified whether total or active GLP-1 was measured [203]. The outcomes from the studies investigating the effects of the polyphenols on the GLP-1 postprandial secretion responses are controversial, probably due to diverse design and experimental conditions: sample size, presence of disease and its stage, meal used to trigger GLP-1 secretory response (quantity and type of the utilized carbohydrates), and way of administration of polyphenols. Further studies will be needed to elucidate the potential of polyphenols to modulate GLP-1 food-triggered secretion response as their possible antidiabetic mechanism of action.

1.2.4 Conclusions: Effects of Pycnogenol® on GLP-1 (active) serum levels

A clear tendency for the expected increased active GLP-1's concentrations between 0 and 30 minutes upon both OGTTs was observed. This tendency was not statistically significant probably due to high data variability.

Intake of PYC for two weeks affected neither the fasting intact GLP-1 serum levels, nor those at 30 minutes upon glucose or boiled rice-equivalent charge.

1.3 Effects of Pycnogenol® on DPP IV serum levels and activities

1.3.1 Background: DPP IV serum concentration and activity

Dipeptidyl peptidase IV (DPP IV, CD26, ADCP2) is a serine exopeptidase that cleaves N-terminal dipeptides from diverse substrates, among which are some important signaling molecules. These include the incretin hormones GLP-1 and GIP, which, as mentioned before, play a major role in the regulation of insulin secretion and respectively the glucose metabolism. DPP IV is expressed in varieties of organs and tissues, e.g. in brush-border membranes of small intestine, liver, pancreas, endothelium of the capillaries. DPP IV exists both as a membrane-bound enzyme, and as a soluble form in blood plasma, the latter lacking the cytoplasmic and transmembrane domains of the protein [204]. DPP IV cleaves GLP-1 and thereby terminates its effect on insulin release. This is clinically important especially in type II diabetes mellitus patients (T2DM) because a positive correlation between the HbA1c levels and DPP IV activity has been established [195]. For this reason, DPP IV inhibition is a promising strategy in diabetes treatment. Multiple DPP IV inhibitors (sitagliptin, saxagliptin, vildagliptin) have already been approved as drugs [205]. Briefly, the capability of DPP IV to deactivate the incretin hormones coupled with DPP IV-induced impairment of insulin sensitivity and signaling make the enzyme an important target for treatment of T2DM. There is an extensive ongoing research for discovery and development of novel molecules that decrease secretion, plasma levels, and inhibit activity of this enzyme.

1.3.2 Results: Effects of Pycnogenol® on DPP IV serum levels and activities

1.3.2.1 Serum concentrations of DPP IV in healthy individuals

As described in B-1.2.2.3 the samples of T1-0 and T2-0 were combined as a "control state", and T3-0 and T4-0 represented a "treated state". There was a decrease in the mean levels of soluble serum DPP IV in treated state (T3, T4; n= 38) compared to the control

one (T1, T2; n= 38) by 8.2 %, which was statistically significant (p= 0.032, two tailed paired Student's t-test). Of the 40 samples per group 4 values (13-T2-0, 13-T4-0, 15-T4-0, and 17-T2-0) were identified as outliers (> 2.5 S.D.) using a box plot test [191] and were excluded from the statistical test data analysis. Individual DPP IV levels are listed in the Supplement, Section D-1.2.1.

The mean DPP IV concentration of the treated state (T3, T4) of 690.91 ± 246.12 ng/mL was lower than the mean value of the control state (T1, T2) of 752.60 ± 227.48 ng/mL (Figure 14.1a).

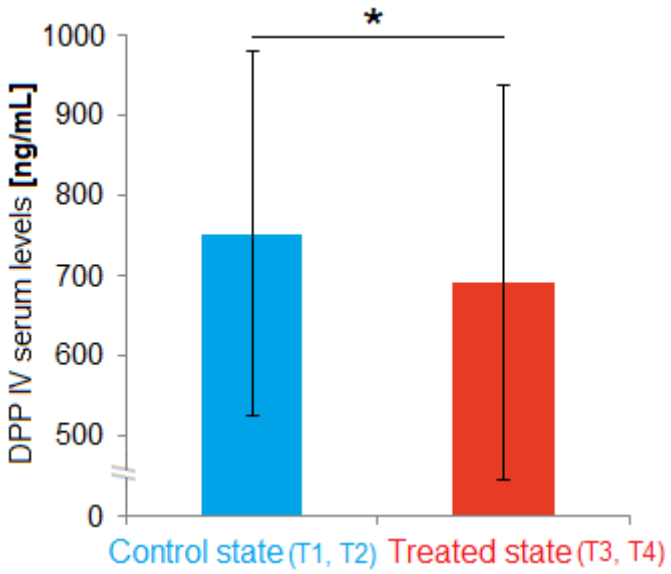


Figure 14.1a: Mean serum concentrations of DPP IV in healthy individuals before and after 2 weeks of PYC intake (100 mg/day). Columns represent means and standard deviation (n= 38). The difference of 8.2 % was statistically significant (p= 0.032).

The mean values, standard deviations (S.D.), medians, mean deviations (Mean Dev.) are listed in the Supplement, Section D-1.2.1.

Additionally, the individual changes in DPP IV serum concentrations for each volunteer between states T1-T3 and T2-T4 were calculated (in %). The absolute values were normalized to the mean of the control state. The mean of the individual changes of all of the samples included into the analysis revealed a decrease in DPP IV concentrations of 10.71 % after PYC intake. This change was statistically significant (p= 0.0003, two tailed paired Student's t-test, n= 36). Along with the absolute values shown previously, two normalized ratios (16-T1-0/T3-0, 16-T2-0/T4-0) were detected as outliers as described above and were excluded from the data analysis. The values of individual change for each participant are listed in the Supplement, Section D-1.2.1. The mean of treated state (T3, T4) of 89.29 ± 16.06 % was lower than the mean of control state – 100 ± 31.10 % (Figure 14.1b).

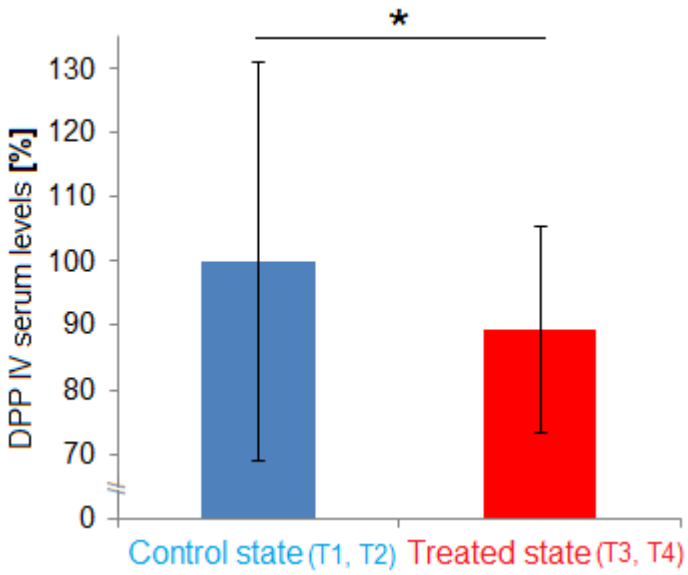


Figure 14.1b: Serum concentrations of DPP IV in healthy individuals before and after 2 weeks of PYC intake (100 mg/day). Columns represent means (control state = 100 ± 31.10 %, treated state = 89.29 ± 16.06 %) and standard deviation (n= 36) of the normalized individual changes in DPP IV serum concentrations. The mean of individual differences showed decrease of 10.71 % (p= 0.0003) after statistical exclusion of outliers. The absolute values were normalized to the mean of the control state, * p< 0.05.

1.3.2.2 Serum activities of DPP IV in healthy individuals

There was no difference between the baseline of control (T1, T2) and treated states (T3, T4) regarding the mean DPP IV activity in the group of healthy individuals (n= 39). Two outliers (20-T3-0 and 2-T2-0) were detected and excluded from the descriptive statistics and further statistical test data analysis as described above. The mean of control state (T1, T2) was 745.41 ± 131.35 µU/mL and the mean of the treated state (T3, T4) was 742.47 ± 160.12 µU/mL (Figure 14.2a). For the individual DPP IV activities please refer to Supplement, Section D-1.2.2.

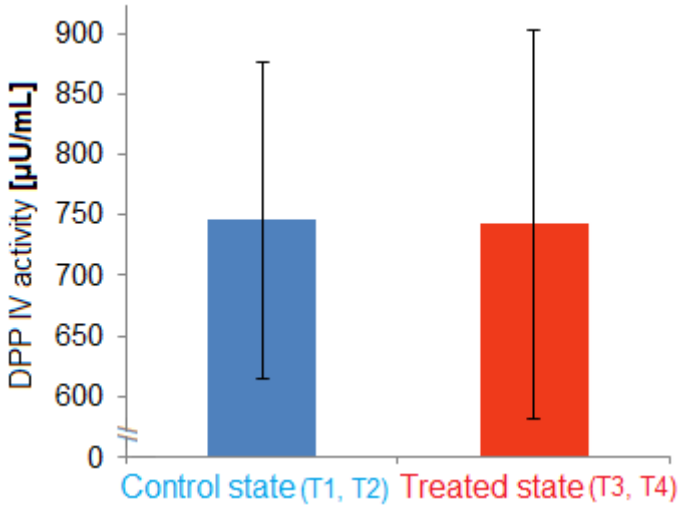


Figure 14.2a: DPP IV activity in serum probes of healthy individuals before (T1, T2) and after 2 weeks of PYC intake (100 mg/day) (T3, T4). Columns represent mean and standard deviation (n= 39). No distinct differences were observed.

The mean values, standard deviations (S.D.), medians, mean deviations (Mean Dev.) are listed in the Supplement, Section D-1.2.2.

Additionally, the individual changes of serum DPP IV enzymatic activities for each participant between states T1-T3 and T2-T4 were calculated. Along with the absolute values shown above (20-T3-0 and 2-T2-0), five normalized ratios outliers (11-T1/T3, 14-T1/T3, 1-T2/T4, 13-T2/T4, and 15-T2/T4) were detected as described above and were excluded from further analysis. The mean of the individual changes of all of the samples included in the analysis indicated a slight, but not statistically significant (p= 0.25) decrease in DPP IV activity of 3.17 % after PYC intake. The values of the individual change of DPP IV activity for each volunteer are listed in the Supplement, Section D-1.2.2. The absolute values were normalized to the mean of the control state: the mean value of the control state (T1, T2) (100 ± 17.78 %) was slightly higher than the mean value of the treated state (T3, T4) (96.83 ± 15.59 %) (Figure 14.2b):

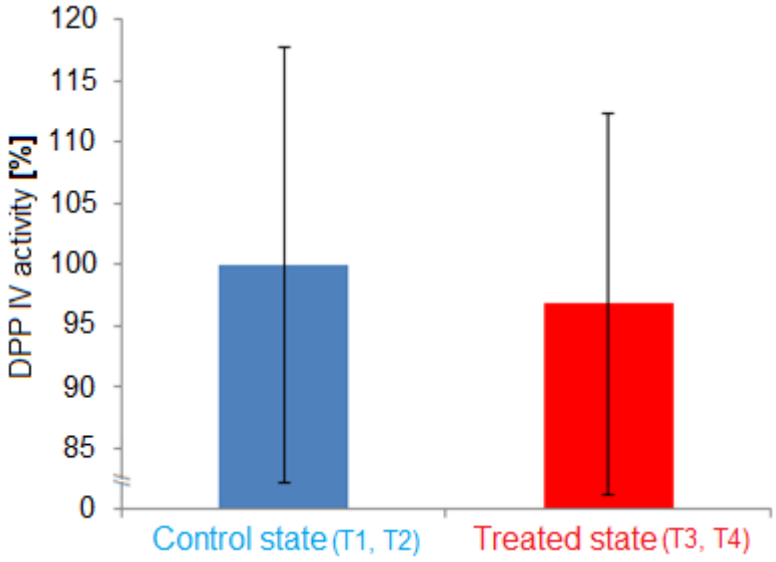


Figure 14.2b: DPP IV activity in serum samples of healthy individuals before (T1, T2) and after 2 weeks of PYC intake (100 mg/day). Columns represent mean and standard deviation of the normalized individual values of DPP IV serum activities (control state = 100 ± 17.78 %, treated state = 96.83 ± 15.59 %) (n = 34). The mean of individual differences showed small and not statistically significant decrease of 3.17 % after PYC-intake (p= 0.25) after statistical exclusion of outliers. The absolute values were normalized to the mean of the control state.

1.3.3 Discussion: Effects of Pycnogenol® on DPP IV serum levels and activities

To the best of our knowledge, the present study is the first one that investigated the effects of a plant-derived extract (namely PYC) on the serum concentrations of soluble DPP IV/ CD26 and its serum enzymatic activity in humans. The data indicated that two weeks of PYC intake (100 mg/day) statistically significantly (p= 0.0003, n= 36) decreased serum concentrations of DPP IV by 10.71 % in healthy individuals. The mean serum concentrations of DPP IV ranged from 653.79 to 755.82 ng/mL and were similar to the concentrations obtained in other studies with healthy participants. For instance, Lamers et al. reported DPP IV concentrations in healthy individuals of 200 ng/mL to 600 ng/mL [206]. Cordero OJ et al. observed serum concentrations of 590 ± 81 ng/mL in healthy participants [207, 208]. Higher concentrations in individuals without presence of a disease were also found (median 1427 (1080-1620) ng/mL) [209]. These variances can be explained with differences in cohorts' characteristics and with different immunoassays utilized.

Surprisingly, despite lower DPP IV serum concentrations after 2-weeks long PYC-intake no difference between the mean of DPP IV activities between control and treated states was detected. The mean of individual changes revealed only slight and statistically non-significant decrease of 3.17 %. It appears to be contradictory that the decrease in serum concentrations of the enzyme is not paralleled by a decrease in DPP IV activity. Indeed, there is a report on positive correlations between DPP IV/CD26 serum concentrations and activities [210]. However, other authors discussed a lack of direct correlation between serum concentrations and activities of DPP IV and suggested various reasons [208]. Briefly, some other circulating enzymes different than DPP IV possess the same activity and are called DASH (DPP IV activity and/or structure homologs) proteins [211]. Additionally, neutral endopeptidase 24.11 was shown to degrade GLP-1 *in vivo*, but its importance was shown to be secondary [212]. Therefore, activities of the enzymes mimicking DPP IV should be taken into consideration. However, in the present assay, sample blanks (C-1.4.5), considering such non-DPP IV activities, were included and subtracted from the respective values. In addition, posttranslational modifications as hypersialylation might inhibit DPP IV activity. Thus, another explanation of the present results might be the presence of an inactive form of DPP IV that contributed to the serum concentrations, but not to the enzymatic activity. A further possibility would be that probably the reduction in serum concentrations of the enzyme is not sufficient to influence notably its activity. However, still the observed decrease of DPP IV concentrations might also be important *per se* as DPP IV was recently identified as a novel signaling molecule (adipokine), which is capable to impair insulin signaling and probably plays an important role in metabolic syndrome pathophysiology [206].

The present data indicated a slight but statistically significant decrease in DPP IV concentrations in healthy individuals, while the activities of the enzyme remained unchanged. The decrease in the concentrations is not expected to contribute to adverse events, such as hypoglycemia, as studies suggested that the application of the DPP IV inhibitor sitagliptin in healthy humans is safe with no reported significant impact on fasting plasma glucose or glucose tolerance [213].

However, in contrast to healthy humans the DPP IV activity in serum from T2DM is elevated [195, 214]. Thus, if PYC is capable to decrease serum concentrations or activity of DPP IV in T2DM patients, this might be beneficial and contribute to antidiabetic effects.

Although there are various *in silico* an *in vitro* studies investigating the potential of plant extracts and their constituent polyphenols to inhibit DPP IV activity [215-217], the results should be interpreted carefully. In order to evaluate the applicability of the studies in humans, the physiological relevance of plasma concentrations and bioavailability, along with the biotransformation of the polyphenolic compounds must be considered. Typically, plasma concentrations of total (active and inactive) metabolites of a given polyphenol after ingestion of 50 mg of its aglycone equivalents are in range 0 to 4 µM (often not more than

2 µM) [218]. In accordance, in a pharmacokinetic study of PYC constituents (A-3.5.2) and metabolites have been detected in nM range (i.e. catechin 60 ng/mL (0.21 nM), ferulic acid 10-15 ng/mL (0.051 – 0.077 nM), and caffeic acid 5-10 ng/mL (0.028 – 0.056 nM)) [165]. Such concentrations are characteristic and physiologically relevant in humans.

The concentrations used in *in vitro* approaches are usually much higher than that detected in human plasma [219, 220]. Anyway, some *in vitro* biochemical assays revealed that certain polyphenols inhibited DPP IV at low micromolar concentrations, e.g. cirsimaritin (IC_{50} = 0.43 ± 0.07 µM), hispidulin (IC_{50} = 0.49 ± 0.06 µM), and naringenin (IC_{50} = 2.5 ± 0.29 µM) [221]. Combined *in vitro/in silico* approaches reported inhibition of DPP IV by resveratrol (IC_{50} = 0.6 ± 0.4 nM), luteolin (IC_{50} = 0.12 ± 0.01 µM), apigenin (IC_{50} = 0.14 ± 0.02 µM), flavone (IC_{50} = 0.17 ± 0.01 µM) [215], and cyanidin 3, 5-diglucoside (IC_{50} = 5.5 µM) [222]. The results from the latter studies indicated that some polyphenols might have a potential as DPP IV inhibitors.

The findings from *in silico*, *in vitro*, and *in vivo* studies for inhibitory effect of some polyphenols and plant extracts on the enzymatic activity and expression of DPP IV demand interventional clinical studies in order to confirm or disapprove their therapeutic potential.

The lack of a significant decrease in DPP IV activity after PYC supplementation is consistent with the results for intact GLP-1 levels B-1.2.2, where no significant influence of PYC on the serum levels of the incretin hormone was detected. Clinical studies investigating the effects of PYC on DPP IV concentrations and activity within T2DM patients would be helpful to confirm the role of DPP IV in the antidiabetic effect of PYC.

1.3.4 Conclusions: Effects of Pycnogenol® on DPP IV serum levels and activities

The mean DPP IV serum concentrations after PYC-intake period (T3, T4) were lower than the mean concentrations determined at the control state (T1, T2) and this difference was statistically significant (p= 0.032). However, there was no indication that PYC significantly decreased the serum enzyme activity of DPP IV in the investigated participants.

1.4 Effects of Pycnogenol® on relaxin-2 serum concentrations

1.4.1 Background: Relaxin-2 serum levels

Relaxin-2, a representative of the insulin superfamily hormones, has been investigated for its potential to improve β-cells' function and insulin sensitivity in T2DM models and human patients [223]. In this respect the present study aimed to investigate, whether PYC-intake might influence the serum concentrations of relaxin-2 in human volunteers. As no evidence exists that relaxin-2 is impacted by food intake all of the measurements have been performed with serum samples obtained at 120 minutes after OGTT.

1.4.2 Results: Effects of Pycnogenol® on relaxin-2 serum levels

In the present study serum relaxin-2 concentrations have also been measured, using one of the most sensitive and specific (utilizing monoclonal antibody) immunoassay kits available on the market (C-1.2.1). All of the measured samples had concentrations below the lowest standard, 7.8 pg/mL and most of them were even beneath the minimum detectable concentration (1.00 pg/mL). Thus they were considered below the limit of detection (Supplement, Section D-1.3).

1.4.3 Discussion: Effects of Pycnogenol® on relaxin-2 serum levels

Zhang et al. [224] reported relaxin-2 concentrations in healthy individuals in the range of around 50 pg/mL, which was not confirmed in the present study. Possible explanation for the remarkably different previous result [224] can be the specificity of the employed antibodies. Indeed, Whittaker et al. warned about the possibility that some immunological assays may be detecting relaxin-like substances different than the supposed human relaxin-2 antigen, leading to readouts higher than the real ones [225].

1.4.4 Conclusions: Effects of Pycnogenol® on relaxin-2 serum levels

Relaxin-2 serum levels in the serum samples of the investigated participants were beneath the limit of detection (< 7.8 pg/mL). No conclusions could be drawn.

1.5 Effects of Pycnogenol® on serum adiponectin

1.5.1 Background: Adiponectin serum levels

Another molecule that plays a key role in the pathogenesis of T2DM and its related metabolic disorders is adiponectin. Adiponectin is a polypeptide which exerts endocrine and paracrine signaling activities. It belongs to the group of adipocytokines – signaling molecules excreted by the adipose tissue which has been considered as an important endocrine organ [226]. Adiponectin (AdipoQ, Acrp30) is highly abundant in plasma, in humans it has been detected in µg/mL range. Characteristic are also gender differences: generally females have higher adiponectin levels than males. It was also reported that adiponectin plasma concentrations are lower in individuals with obesity, as well as in patients with insulin resistance and T2DM in comparison to healthy persons [227]. Adiponectin's functions are primarily related to metabolic homeostasis – alleviation of insulin resistance, upregulation of glucose intake, and fatty acids combustion. On cellular level these effects are mediated by adiponectin receptors (Adipo1R and Adipo2R) which activate AMPK (AMP-activated protein kinase, A-2.2) and PPARα (peroxisome proliferator-activated receptor alpha) [228]. In addition, adiponectin appears to act as anti-inflammatory cytokine [229], ameliorate endothelial dysfunction and is reported to display protective effects on cardiovascular system [230]. The variety of beneficial effects discussed for adiponectin make it a promising target in therapy of T2DM and metabolic syndrome. Research activities investigate for instance how adiponectin can be modulated in means of physiology, nutritional science, and pharmacology [231]. There are also efforts for development of synthetic AdipoR-agonists [232]. Several clinical studies [233-239] indicated the potential of natural compounds and especially polyphenols [236-239] to elevate adiponectin serum concentrations.

Information about the effects of meal or glucose intake on total adiponectin levels is controversial. Some authors suggested no influence on serum adiponectin concentrations. [240-242]. In contrast, postprandial changes in total adiponectin levels have been observed by others [243, 244]. Therefore in the present study possible responses in adiponectin concentrations to glucose or boiled rice equivalent was examined.

1.5.2 Results: Effects of Pycnogenol® on adiponectin serum levels

PYC caused a very slight and statistically non-significant increase in the mean fasting adiponectin concentrations, in comparison to the mean fasting concentrations before PYC-intake. Four values (16-T1-0, 16-T2-120, 16-T3-0, and 16-T3-120) were identified as outliers (as indicated in C-1.5.3) and were subsequently excluded from the analysis. The values of the measured concentrations can be found in the supplementary attachment (Supplement, Section D-1.4).

1.5.2.1 Classic OGTT protocol

The present data suggest that the means of total adiponectin concentrations increased slightly and non-statistically significantly at 120' after oral glucose charge in comparison to fasting state both before and after PYC-treatment (Figure 15.1). Before PYC intake the mean increase was 0.93 µg/mL (T1-0: 7.38 ± 1.80 µg/mL (n= 19) and T1-120: 8.31 ± 2.78 µg/mL (n= 20)). After PYC intake the mean increase was 0.16 µg/mL (T3-0: 7.43 ± 2.25 µg/mL (n= 19) and T3-120: 7.59 ± 2.38 µg/mL (n= 19)). Thus, the increase of the mean concentrations was higher before PYC-intake [ΔT1 (0-120) = 0.93 µg/mL] compared to the increase after PYC-intake [mean ΔT3 (0-120) = 0.16 µg/mL]. A tendency for very small increase in the mean fasting adiponectin concentrations after PYC-intake, in comparison to the mean fasting concentrations before PYC-intake (Δ (T1-0/T3-0) = 0.05 µg/mL) was observed. None of these differences between the group means were statistically significant (p= 0.58, one-way ANOVA, n= 19 for T1-0, T3-0, T3-120, n= 20 for T1-120).

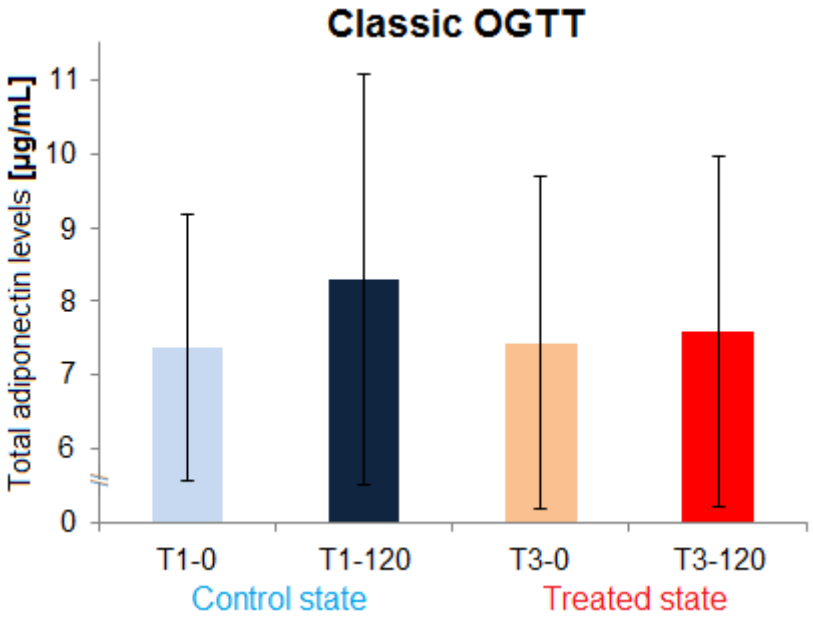


Figure 15.1: Total adiponectin levels in healthy individuals before (T1-0, T1-30 – Control state) and after 2 weeks of PYC supplementation (100 mg/day) (T3-0, T3-30 – Treated state). Columns represent means and standard deviation. Glucose OGTT induced slight rise in adiponectin concentrations compared to the fasting concentrations, both in control [ΔT1 (0-120) = 0.93 µg/mL] and treated state [mean ΔT3 (0-120) = 0.16 µg/mL], which was not statistically significant (p= 0.58, one-way ANOVA, n= 19 for T1-0, T3-0, T3-120, n= 20 for T1-120).

The mean values, standard deviations (S.D.), medians, and mean deviation (Mean Dev), are listed in the Supplement, Section D-1.4.

1.5.2.2 Boiled rice equivalent OGTT protocol

Boiled rice OGTT induced different adiponectin response pattern compared to glucose. In the control state before PYC-intake, rice OGTT caused a slight though not statistically significant decrease of 0.61 µg/mL in the mean adiponectin levels at 120 minutes (T2-120 = 7.66 ± 1.91 µg/mL (n= 19)) compared to the fasting adiponectin levels (T2-0 = 8.27 ± 2.66 µg/mL (n= 20)). In contrast, in the treated state after PYC intake, rice OGTT increased slightly (but not statistically significantly) adiponectin concentrations at 120 minutes (T4-120 = 8.81 ± 3.29 µg/mL (n= 20)) by 0.32 µg/mL compared to the fasting adiponectin levels (T4-0 = 8.49 ± 3.18 µg/mL (n= 20)). Similarly to classic OGTT protocol, a slight and non-statistically significant tendency for increase (0.22 µg/mL) in basal adiponectin concentrations in treated state (T4-0) compared to the control one (T2-0) was observed (Figure 15.2).

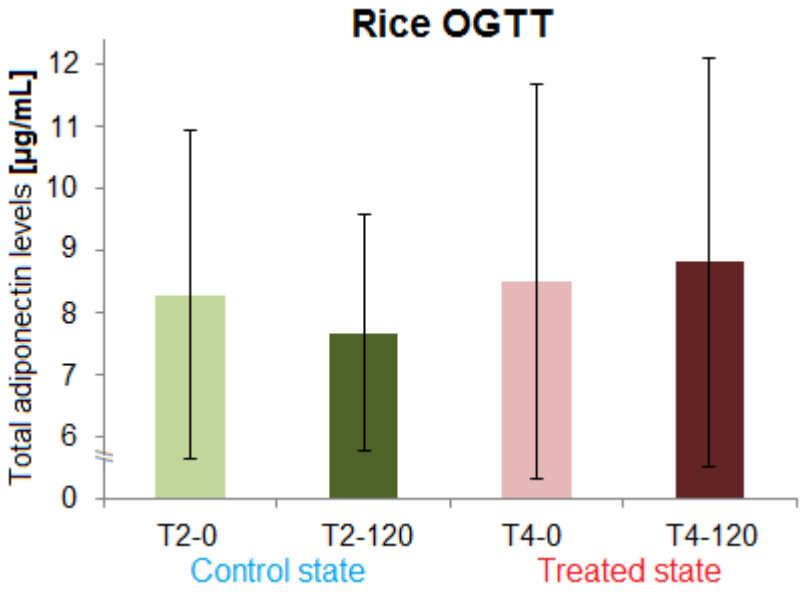


Figure 15.2: Serum concentrations of total adiponectin in healthy individuals before (T2-0, T2-120) and after 2 weeks PYC intake (100 mg/day) (T4-0, T4-120). Columns represent means and standard deviation. Rice OGTT caused slight, but not statistically significant decrease [ΔT2 (0-120) = –0.61 µg/mL] in total adiponectin levels (compared to fasting adiponectin levels) before PYC intake. Conversely, after PYC treatment, rice OGTT caused slight, but not statistically significant increase in adiponectin levels [ΔT4 (0-120) = 0.32 µg/mL] compared to the respective fasting levels (p= 0.64, one-way ANOVA; for T2-0, T4-0, T4-120 – n= 20; for T2-120 – n= 19).

The mean values, standard deviations (S.D.), medians, mean deviation (Mean Dev), and standard error of the mean (S.E.M.) are listed in Supplement, Section D-1.4.

1.5.2.3 Individual changes of adiponectin

Additionally, the individual changes of serum adiponectin concentrations for each participant between the study phases T1-T3 and T2-T4 were calculated (Figure 15.3).

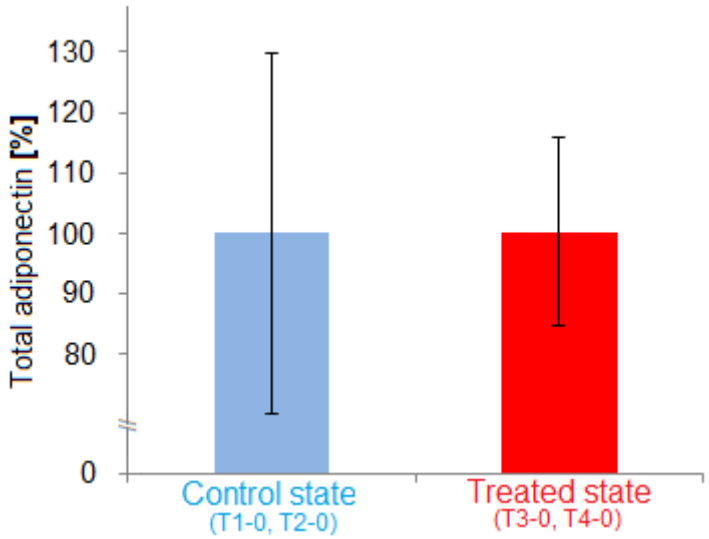


Figure 15.3: Individual changes of basal adiponectin concentrations in healthy volunteers before (T1-0, T2-0) and after 2 weeks PYC intake (100 mg/day) (T3-0, T4-0). Columns represent means and standard deviation. The mean of the individual changes indicated no differences before and after PYC-intake period (n= 38). The absolute values were normalized to the mean of the control state.

The samples of T1-0 and T2-0 were combined as a "control state" and those of T3-0 and T4-0 were combined as a "treated state" (as in B-1.2.2.3). The absolute values were normalized to the mean of the control state (100 %). Along with 16-T1-0 and 16-T3-0, their normalized ratio 16-T1-0/T3-0 and 8-T2-0/T4-0, were detected as outliers as described above and were excluded from further data analysis. The mean of individual changes revealed no difference between control and treated state. The mean of control state of 100 ± 29.61 % did not significantly differ from the mean of the treated state 100.19 ± 15.63 % (n= 38, p= 0.94, two tailed paired Student's t-test). The values of individual changes are documented in the Supplement, Section D-1.4.

Individual changes of adiponectin upon OGTTs

In order to evaluate the individual adiponectin response to glucose or rice load in healthy participants, the individual changes between fasting concentrations at 0 and 120 were analyzed. The individual changes revealed no statistically significant adiponectin responses to glucose/rice challenges, neither before nor after PYC-intake. The single values of the individual changes are documented in the Supplement, Section D-1.4.

1.5.3 Discussion: Effects of Pycnogenol® on serum adiponectin

The obtained results revealed slight and non-statistically significant changes in adiponectin levels as a response to glucose or boiled rice charge. The mean total adiponectin concentrations ranged between 7.38 and 8.81 µg/mL were similar to the ones reported in previous studies with healthy participants 10.34 ± 4.59 µg/mL – females; 8.04 ± 5.10 µg/mL – males [245], 8.81 ± 3.43 µg/mL [246]. The difference in serum concentrations between men and women in the present study were not evaluated because there was no information about the gender of the volunteers available.

As mentioned before the information about postprandial changes in adiponectin levels is controversial. In the present study there were only slight and not statistically significant variations in total adiponectin levels as responses to classic and rice OGTT. The present study confirmed the statement from previous investigations that there is no statistically significant serum adiponectin response to glucose/rice challenges.

The mean values of basal adiponectin levels (T1-0, T2-0) tended to increase after PYC intake (T3-0, T4-0), which was observed both in classic OGTT protocol (7.38 to 7.43 µg/mL) and in the boiled rice one (8.27 to 8.49µg/mL). These changes were minor and not statistically significant. Although the mean of individual changes revealed no difference between control and treated states, bigger sample size might be useful to detect whether these differences are consistent or not. Increase in adiponectin levels are generally desired, as this adipokine exerts various beneficial effects in metabolic syndrome and diabetes, such as anti-inflammatory, anti-atherosclerotic, and alleviating insulin resistance [247].

1.5.4 Conclusions: Effects of Pycnogenol® on serum adiponectin

The adiponectin concentrations responded slightly, but not statistically significantly to the glucose and boiled rice-equivalent challenge, but not to the intake of PYC. Bigger sample-size might reveal whether these changes have any clinical significance.

2 Effects of polyphenols on Akt-kinase-phosphorylation

2.1 Effects of polyphenols on Akt-phosphorylation – pilot study

2.1.1 Background

Akt-kinase (also known as Protein Kinase B, PKB) plays a crucial role in mediating the insulin effects on the intracellular level and is therefore important in the pathophysiology of diabetes and its vascular complications [20]. There is evidence that the Akt-signaling pathway might be altered in T2DM and this might contribute to insulin resistance [248]. In addition, this kinase can affect further processes associated with T2DM and its long-term consequences. These include glucose transport, glycogen synthesis [249], apoptosis [34], endothelial dysfunction [11], and angiogenesis [250]. Therefore, modulators of Akt-phosphorylation and respectively activity are extensively investigated as pharmacological tools [59]. They can be advantageous for basic and translational research addressing diabetes mellitus and its vascular complications.

As already described under A-2.1.3, Akt can be activated through phosphorylation at various sites among which the most important are the amino acids threonine 308 (Thr308) and serine 473 (Ser473).

Akt has a key role in the cardiovascular system. In general, Akt1 isoform is predominantly expressed in the endothelium. It has been reported that the activation of Akt-kinase in endothelial cells (EC) contributes to the cardio-metabolic homeostasis by subsequent activation of eNOS and release of NO, and possesses protective properties in relation to long-term vascular complications of T2DM [63] (see also A-2.1.5).

Various *in vitro* [138], *in vivo* [251-253], and clinical studies [254-256] suggested that polyphenols might have beneficial effects on diabetes-induced late complications on the cardiovascular system. In certain cases it was described that these effects are mediated through Akt-kinase [251, 252].
The potential relationship between the beneficial health promoting properties of polyphenolic compounds and their capability to modulate insulin signal transduction demanded a comprehensive study regarding the effects of individual polyphenols on the phosphorylation of Akt-kinase.

2.1.2 Results: Pilot study

Effects of quercetin and resveratrol on the phosphorylation of Akt at Ser473 site (pAkt Ser473) were analyzed by Western blot (C-2.4.4). The experiments indicated that a short incubation (30 minutes) caused statistically significant inhibition on pAkt Ser473 (mean ± standard deviation) which was 37.79 ± 7.14 % (p= 0.021, n= 4) for resveratrol and 52.37 ± 21.01 % (p= 0.006, n= 3) for quercetin (one-way ANOVA with Tukey's post-hoc test) (Figure 16, A). The effect of resveratrol was also investigated for another short-term treatment time-point (15 minutes) and was very similar (39.89 ± 23.06 %) and likewise statistically significant (p= 0.036, n= 4, two-tailed, paired Student's t-test). The observed inhibition effects occurred even after shorter incubation of 5 minutes (see primary screening: B-2.2.2). On the other hand, after prolonged incubation both compounds showed a tendency for an increase of Akt-phosphorylation, which was more pronounced for resveratrol (Figure 16, B).

Figure 16: Effects of resveratrol (RSV) and quercetin (Que) on the phosphorylation of Akt-kinase under hyperglycemic (standard cell culture) conditions. Representative Western blot. A. In case of 30 minutes incubation RSV and Que inhibited the phosphorylation of Akt (pAkt Ser473) statistically significantly [p(RSV)= 0.021, n= 4 and p(Que)= 0.006, n= 3, one-way ANOVA]. B. On the contrary, after a longer-term incubation (21 hours) both compounds tended to increase Akt-phosphorylation. Effects of the compounds were normalized to the respective controls. Beta actin served as a loading control.

An overview of the results from the pilot study can be found in the Supplement, Section D-2.1.

2.1.3 Discussion: Pilot study

It was previously described that resveratrol [257-259] and quercetin [260, 261] are capable of improving an existing endothelial dysfunction which in some cases involved an activation of the Akt/eNOS signaling pathway [257, 261]. Contrary to the expectations, the present results revealed that the investigated model compounds at a 10 µM concentration statistically significantly ($p < 0.05$) reduced Akt-phosphorylation after 30 minutes compared to the untreated controls in the employed cell-based model.

On the other hand, quercetin had been reported to inhibit PI3K [262] and tyrosine kinase [159]. These kinases are up-stream of Akt and can regulate its phosphorylation [263]. In accordance with that it had been reported that 10 µM quercetin inhibited eNOS activity by inhibiting Akt-signaling in bovine aortic endothelial cells (BAEC) [264]. Likewise, resveratrol was reported to inhibit PI3K/Akt-pathway [265]. However, the information about effects of resveratrol on Akt-phosphorylation in endothelial cells is limited. Kanavi et al. [266] reported that 100 µM resveratrol downregulated the phosphorylation of Akt in choroidal endothelial cells. In et al. [267] described that high concentrations of resveratrol (also 100 µM) inhibited Akt/eNOS signaling, and therefore it was considered as a potent anti-angiogenic substance. In F-2 endothelial cells 100 µM resveratrol activated eNOS, while lower concentrations (20 µM) did not induce activation, but antagonized VEGF-induced eNOS-phosphorylation [268]. The activation of eNOS by VEGF is described to be Akt-dependent process [269]. There are contradicting reports that demonstrated Akt-activation by resveratrol and quercetin [257, 261, 270, 271], others indicated Akt-inhibition by both polyphenols [264, 266]. Possible reasons for these discrepancies might be differences in experimental models and conditions: i.e. use of immortalized cell line versus primary cells, intact endothelial monolayer, or employment of *in vivo* or *ex vivo* models. The pilot experiments were performed with immortalized endothelial cells, which are widely used as a standard *in vitro* model. Prior the treatments cells were serum-deprived, as the presence of growth factors might mask some activator effects on the protein phosphorylation. Another important factor for the outcome seemed to be the duration of treatments. The results from the pilot experiments indicated differences in the effects of the investigated compounds: for short incubation polyphenols inhibited Akt-phosphorylation (Figure 16 A), while they showed a tendency to increase Akt-phosphorylation during prolonged incubations (21 h) (Figure 16 B). Possible reasons for this difference might be the chemical instability of the compounds [272] or the fact that they might undergo biotransformation under cell culture conditions [273, 274].

2.2 Effects of polyphenols on Akt-phosphorylation – primary screening

2.2.1 Background: Primary screening

After establishment of the assay which enabled investigations of the phosphorylation status of proteins *in vitro* under normo- and hyperglycemic-like conditions and defining the activity of the model compounds in the pilot study (B-2.1.2), the effects of a higher number of polyphenols with different structures on Akt-kinase were to be characterized. The aim was to identify subclasses and representatives of polyphenols that modulate this signaling pathway and thus might be effective in the prevention and management of T2DM late complications. As inhibitory effects after a short exposure were observed in the pilot study (B-2.1.2), cells were not serum-deprived in order to check if polyphenols were capable to antagonize the growth-factors-induced Akt-phosphorylation and to possibly mimic conditions close to the physiological ones. Due to the chemical instability of polyphenols [272] a short incubation time of 5 minutes was used. Physiologically relevant concentrations (10 µM) [275] were employed. In the primary screening step eight compounds were involved.

2.2.2 Results: Primary screening

The results obtained from the primary screening using Western blot analysis showed that four of eight of the tested polyphenols were able to influence the phosphorylation status of Akt at Ser473 and Thr308 residues in endothelial cells (EA.hy926) under hyperglycemic conditions (25.0 mM glucose). Representatives from three diverse structural subclasses revealed a capability to cause a statistically significant decrease ($p < 0.01$, one-way ANOVA with Tukey HSD test) of Akt-phosphorylation level after short-term treatment (5 minutes) compared to controls.

The compounds with most prominent inhibitory effects on pAkt Ser473 (mean inhibition ± standard deviation) were quercetin (40.46 ± 4.83 %), luteolin (29.96 ± 11.06 %), resveratrol (25.85 % ± 5.72 %) and apigenin (22.57 ± 10.30 %). The following polyphenols caused only a small and statistically non-significant reduction in the phosphorylation of Akt at Ser473 compared to the negative control: genistein (6.77 ± 18.98 %), 3,4',5-trimethoxy-trans-stilbene (3-MS) (8.32 ± 14.16 %). Taxifolin (0.56 ± 17.96 %) did not seem to have any activity (Figure 17).

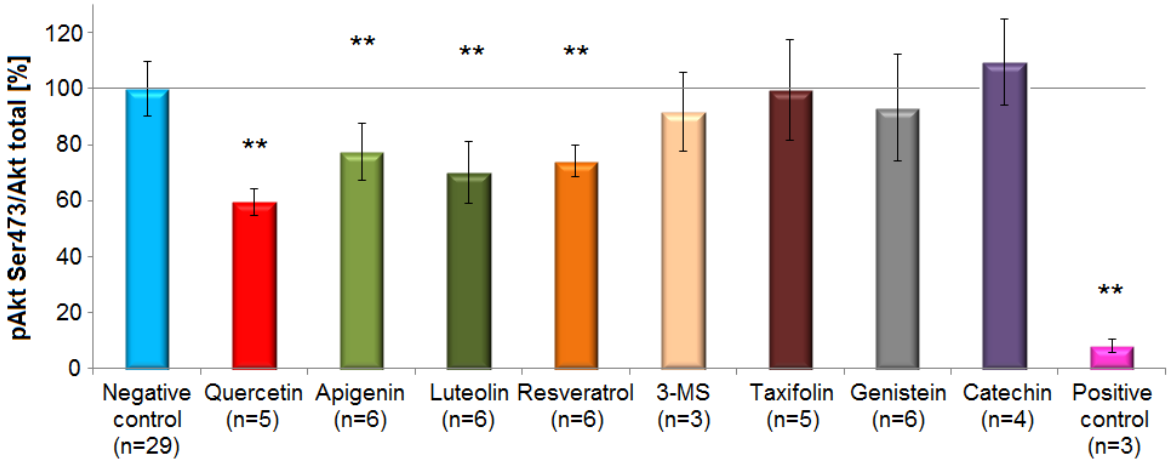


Figure 17: Effects of polyphenolic compounds from different subclasses at a concentration of 10 µM on Akt-phosphorylation at Ser473 site in EA.hy926 cells investigated in the primary screening step using Western blot analysis. Columns represent mean and standard deviation (%). Quercetin (40.46 ± 4.83 %), luteolin (29.96 ± 11.06 %), resveratrol (25.85 % ± 5.72 %), and apigenin (22.57 ± 10.30 %) caused a statistically significant reduction in Akt-phosphorylation compared to the controls (p< 0.01, one-way ANOVA with Tukey HSD test, n= 3-6). On the contrary, 3,4',5-trimethoxy-trans-stilbene (3-MS), taxifolin, genistein and (+)-catechin indicated lack of inhibitory potential.**

The inhibitory effect of polyphenols on the pAkt Thr308 was similar: quercetin (35.71 ± 3.37 %), luteolin (26.90 ± 10.33 %), resveratrol (31.78 ± 13.09 %) and apigenin (28.10 ± 10.21 %).

As a positive control cells were treated with serum-free medium overnight. Under these conditions the phosphorylation level of pAkt Ser473 was strongly reduced by 91.84 ± 2.18 % (n= 3) and the one of pAkt Thr308 by 88.64 ± 5.33 % (n= 3) compared to the respective negative control.

A small, not statistically significant increase in Akt phosphorylation at Ser473 was observed by treatments with (+)-catechin. Similarly, genistein, taxifolin, 3,4',5-trimethoxy-trans-stilbene and (+)-catechin showed minor and no statistically significant effects on Thr308. An overview of the results can be found in the Supplement, Section D-2.2.

Based on the results obtained by Western blot analysis a correlation between the mean inhibitory effects of the tested polyphenols on both phosphorylation sites pAkt Ser473 and pAkt Thr308 was determined. The calculated correlation coefficient was r= 0.9478 (R^2= 0.898) indicating a strong positive and statistically significant (p= 0.0003) correlation between both variables (Figure 18).

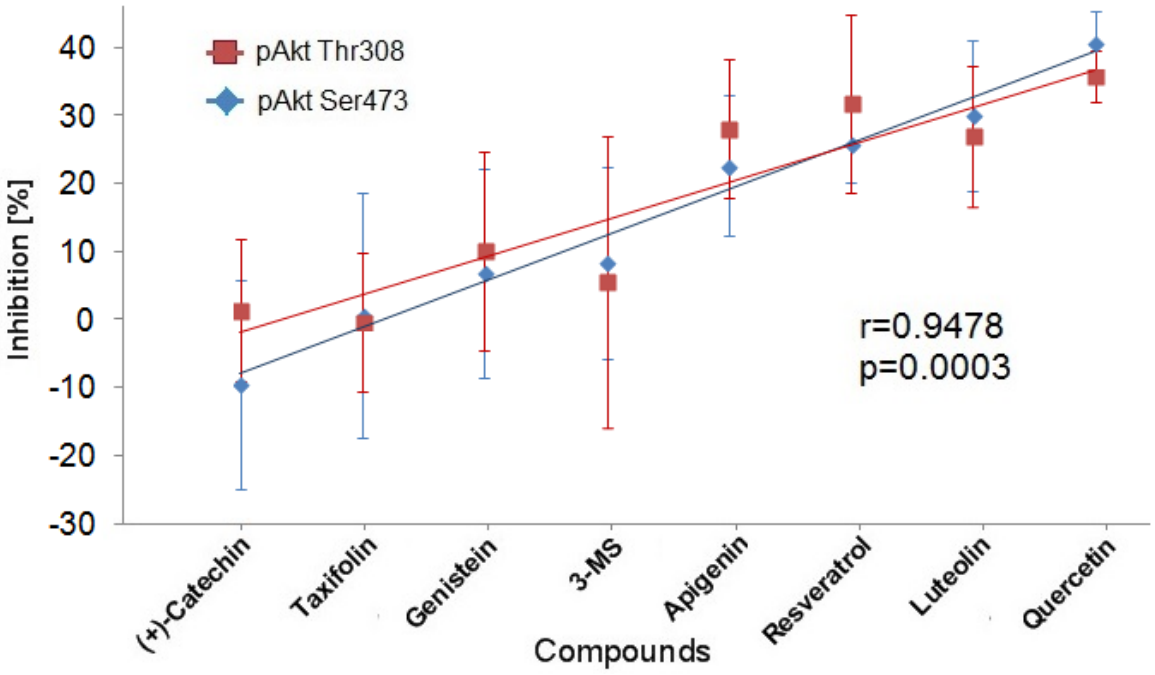


Figure 18: Correlation between the means of Ser473 (blue) and Thr308 (red) phosphorylation statuses of Akt-kinase after treatment with eight different polyphenols included in the primary screening (mean ± S.D.). The tested compounds exhibited similar inhibitory effects on both phosphorylation sites. In accordance with that a strong statistically significant correlation was established (r= 0.9478, p= 0.0003, n= 3-6). 3-MS: 3,4',5-trimethoxy-trans-stilbene

2.2.3 Discussion: Primary screening

Some of the analyzed compounds had moderate, but statistically significant and reproducible effects on the phosphorylation status of Akt. In particular, quercetin, luteolin, apigenin, resveratrol, tested at a physiologically relevant concentration (10 µM) inhibited the phosphorylation of Akt at both amino-acid residues: pAkt Ser473 and pAkt Thr308.

This is consistent with results from previously published studies. It was described that quercetin (concentration not shown) [276] and resveratrol (100 µM) reduced Akt-phosphorylation in endothelial cells (also see B-2.1.3). Likewise, luteolin (10 – 50 µM) [277] and apigenin (1 µM) [278] were reported to reduce Akt-phosphorylation. No reports for the effects of taxifolin and 3,4',5-trimethoxy-trans-stilbene on Akt-phosphorylation in endothelial cells were found. Effects of genistein on the same target were investigated mostly in the context of cancer. However, in a diabetes-relevant study, genistein showed opposite effects on the insulin signaling: either capability to activate or to inhibit Akt. Under normal conditions genistein reduced Akt-phosphorylation in endothelial cells. Conversely, under palmitate-induced insulin resistant conditions genistein promoted

Akt/eNOS activity, probably due to pro-inflammatory pathways suppression. This indicated that the outcome is probably highly dependent on the experimental conditions [279]. To our best knowledge, the present study is the first one to compare (semi-) quantitatively the short-term effects of polyphenols with different structure on Akt-phosphorylation in endothelial cells.

In contrast to most of the published papers, both phosphorylation forms of Akt (Ser473 and Thr308) were investigated in the present study, as both sites are important for the full activation of this enzyme [33]. The mean inhibition effects for each compound on both phosphorylation sites were similar with a strong correlation which had not previously been described. It was therefore conceivable that the polyphenols' effects were achieved by a coordinated mechanism involving both phosphorylation sites, rather than affecting independent up-stream pathways. Vincent et al. postulated that pAkt Thr308 is the more important predictor for the Akt-activity and should preferably be analyzed rather than pAkt Ser473 [280]. The strong correlation found in the present study suggested that both phosphorylation sites were similarly influenced by polyphenols in the utilized *in vitro* model.

Moderate inhibitory effects of polyphenols as observed in the present study might be beneficial in case endothelial dysfunction. It has been described that the hyperactive S6K1 (ribosomal protein S6 kinase beta-1) in senescent endothelial cells might contribute to an increased oxidative stress and decreased NO levels. S6K1 is a downstream target of Akt and its over-activation was reported to contribute to insulin resistance (Figure 19) [281]. It was shown that resveratrol (10 µM) inhibited Akt/S6K1-signaling and reversed endothelial dysfunction and hallmarks of aging [282]. Shew et al. proposed that high-glucose-induced apoptosis in human endothelial cells might involve PI3K/Akt-signaling and blocking of this pathway might attenuate this process [283]. In addition, vascular calcification in diabetes model was also mediated by O-GlcNAcylation-activated Akt-kinase and the osteogenic transcription factor [284].

Akt-kinase has been described as a major regulator of angiogenesis [250, 285] and it was reported that abnormal excessive angiogenesis may contribute to the pathogenesis of microvascular diabetes complications (nephropathy and retinopathy) as well as to atherosclerotic plaque formation [286]. Therefore, it is possible that an over-activation of Akt might play a role in the pathogenesis of these processes. Indeed, Phung et al. reported that sustained activation of endothelial Akt1 may cause aberrant and pathological angiogenesis as well as vascular morphological changes [287]. Furthermore, increased glucose levels were shown contribute to neovascularization in diabetic retinopathy *in vivo*, which was mediated through elevated basal Akt-phosphorylation and inhibition of the latter seemed to prevent the process [288].

According to the literature, Akt-signaling dysregulation is related to diabetes-induced pathological transformations of kidneys. It was proposed that renal hypertrophy and fibrosis as complications of diabetes resulted from high-glucose-activated PI3K/Akt and their downstream target protein p21 (cyclin-dependent kinase inhibitor) [289]. Bai et al. claimed that anti-angiogenic approaches involving VEGF/Akt-inhibition might be successful in the treatment of early diabetic nephropathy [290]. Other researchers proposed Akt-inhibition to be nephroprotective in T2DM [291, 292] and resveratrol was described as a preventive agent [292]. Thus, because of their inhibitory potentials the above-mentioned polyphenols could possess similar advantageous effects.

In addition to the cardiovascular complications a development of cancer has been described as further long-term consequence of T2DM [293]. In the presence of hyperinsulinemia, the Akt-signaling pathway is permanently activated, which contributes to overproduction of reactive oxygen species (ROS) and DNA damage [294, 295]. Metformin, the first-line medication drug for T2DM patients (A-1.6) was described to inhibit Akt-phosphorylation and thereby to prevent insulin-induced overproduction of ROS and the associated with it DNA-toxicity [18]. In this regard, the observed effects of polyphenols on Akt-kinase also might be beneficial in this context (also see B-2.7). Potential beneficial effects of polyphenolic compounds that might be achieved through their inhibitory activity are summarized on Figure 19.

2.2.1 Conclusion: Primary screening

For the first time a study that (semi-)quantitatively compared the influence of polyphenols from six different subclasses on Akt-phosphorylation in endothelial cells was conducted. In addition, both phosphorylated forms Ser473 and Thr308 have been studied since both sites contribute for the complete activation of the kinase. This fact had often been disregarded in papers previously published on similar topics. Quercetin, resveratrol, apigenin and luteolin statistically significantly inhibited the phosphorylation of both Akt Ser473 and Akt Thr308. The inhibitory potential of polyphenols on Akt might be advantageous in case of some T2DM-complications, especially involving pathological neovascularization.

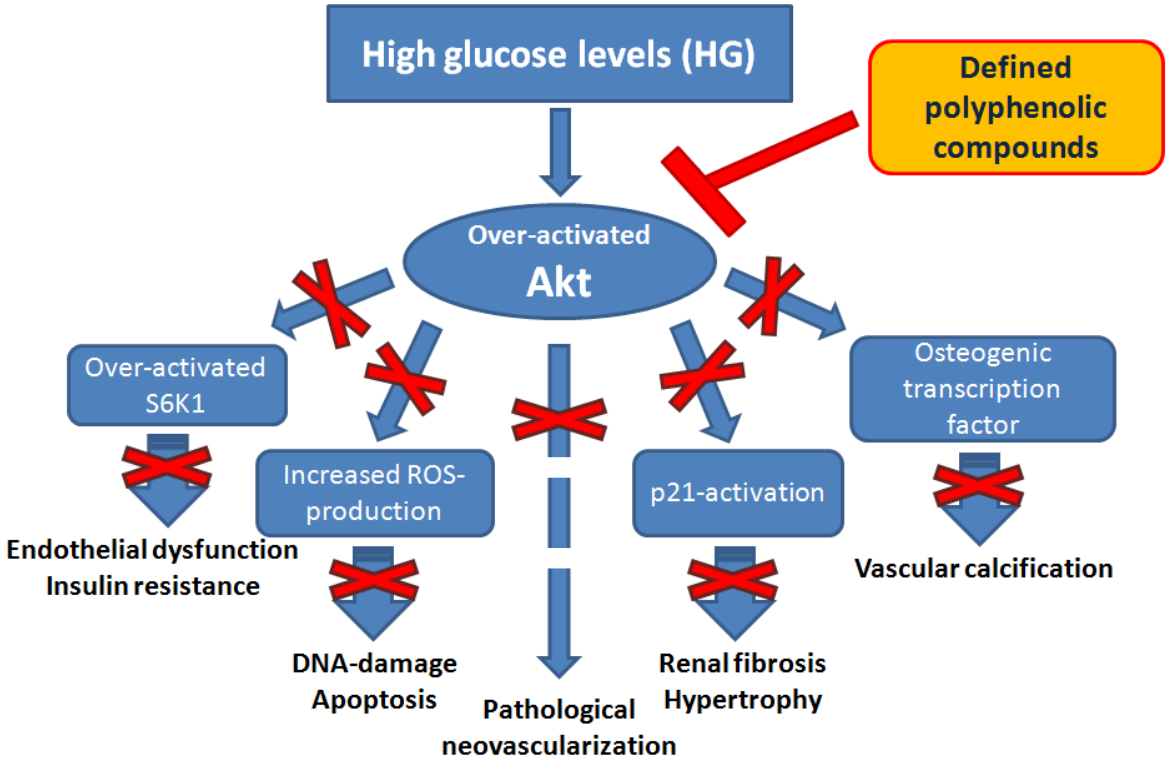


Figure 19: **A summary of proposed potential beneficial effects of polyphenols due to an inhibition of over-activated Akt-signaling pathway. Sustained activation of Akt and its down-stream effectors is related to multiple pathological processes and states like insulin resistance, endothelial dysfunction, aberrant angiogenesis, vascular calcification, DNA-damage, and renal hypertrophy. Inhibition of Akt-phosphorylation by certain polyphenolic compounds might alleviate or prevent these conditions. S6K1: ribosomal protein S6 kinase beta-1; p21: cyclin-dependent kinase inhibitor; ROS: reactive oxygen species.**

2.3 Effects of polyphenols on Akt-phosphorylation – secondary screening

2.3.1 Background: Secondary screening

The primary screening found inhibitory activity on Akt-phosphorylation among the investigated compounds. The most active substances were representatives from the subclasses of flavones, flavone-3-ols, and stilbenoids. A secondary screening was to involve an extended investigation including further representatives of these structural groups, among them glycosylated and methylated derivatives. Additionally, polyphenols and intestinal microbiota-generated metabolites from subclasses that have not been analyzed in the first step were also included (Table 3). The compounds underwent quantitative determination of their effects on the phosphorylation of Akt in endothelial cells *in vitro*.

Table 3.: Polyphenolic compounds and intestinal microbiota-generated metabolites of polyphenols grouped by their chemical structure in subclasses which were included in the secondary screening regarding their *in vitro* inhibitory potential on the phosphorylation of Akt at Ser473 site. In **bold** are indicated subclasses and representatives which had already been involved in the primary screening process. For the classification's source see A-3.2.

Chemical subclass	Individual compounds	Number
Flavones	**luteolin, apigenin,** flavone, 6-hydroxyflavone, 3-hydroxyflavone, 6-methoxyflavone, 7-methoxyflavone, 7,8-dihydroxyflavone, chrysin, baicalein, baicalin, 3-methoxyflavone, 3,4'-dihydroxyflavone, 3-hydroxy-4'-methoxyflavone, vitexin, wogonoside	16
Flavon-3-ols (Flavonols)	**quercetin,** fisetin, kaempferol, myricetin, morin	5
Stilbenoids	**resveratrol**, pinostilbene, pterostilbene, **3,4',5-trimethoxy-trans-stilbene,** piceatannol	5
Flavan-3-ols (Flavanols)	**(+)-catechin, taxifolin,** (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, (-)-gallocatechin gallate	6
Isoflavones	**genistein**	1
Flavanones	naringenin	1
Phenolic acids	caffeic acid, trans-ferulic acid, chlorogenic acid	3
Catechin metabolites	M1, M2	2
Ellagic acid and its metabolites	ellagic acid, urolithin A, B, C, D	5

2.3.2 Results: Secondary screening

The previous results (B-2.1.2 and B-2.2.2) obtained by Western blot revealed that the polyphenols resveratrol, quercetin, apigenin, and luteolin were able to statistically significantly inhibit the phosphorylation of Akt. These inhibitory effects were re-examined by a quantitative analysis through ELISA. The study confirmed their potential to reduce the phosphorylation level of Akt-kinase after a short-term treatment of 5 minutes.

The comprehensive screening regarding inhibitory potentials of other polyphenols and their metabolites on the phosphorylation level of Akt at Ser473 site involved 44 compounds (Table 3). Overall, 26 substances demonstrated some inhibitory potential. Among them 11 substances were characterized to possess pronounced (> 20 %), 10 compounds lower but still distinguishable (between 10 % and 20 %) and 5 substances weak (less than 10 %) inhibitory activity. All other 18 substances caused no inhibition.
Flavones and flavon-3-ols caused the clearest reduction of Akt-phosphorylation. The most active compound was luteolin, followed by quercetin and apigenin. The subclass of flavanols possessed no inhibitory effects with the exception of (-)-gallocatechin gallate. The representatives of flavanones (naringenin), isoflavones (genistein), and flavanonols (taxifolin) showed no considerable inhibition and were not further investigated. Among the group of stilbenoids resveratrol and pinostilbene were active, unlike pterostilbene, 3,4',5-trimethoxy-trans-stilbene and piceatannol which caused no reduction of Akt-phosphorylation. All three structural analogues from the subclass of phenolic acids (caffeic, ferulic and chlorogenic acids) showed no inhibitory effects. Only urolithin A from the group of ellagic acid's metabolites induced an inhibition.

(+)-Catechin, (-)-epicatechin, ferulic acid, M2, naringenin and all investigated glycosides (vitexin, wogonoside, baicalin) exhibited a non-statistically significant tendency to slightly augment Akt-phosphorylation. Detailed information about the effects for all investigated compounds on Akt-phosphorylation is summarized in Supplement, Section D-2.3.

The inhibitory potential of polyphenols was expressed as percentage in comparison to the vehicle-treated negative controls ± standard deviation (100 % – residual phosphorylation). The substances which possessed the most prominent inhibitory effects were luteolin (44.31 ± 17.95 %), quercetin (35.71 ± 8.33 %), urolithin A (35.28 ± 11.80 %), apigenin (31.79 ± 6.16 %), fisetin (28.09 ± 9.09 %), 6-hydroxyflavone (26.86 ± 7.78 %), resveratrol (26.04 ± 5.58 %), 6-methoxyflavone (24.89 ± 10.29 %), flavone (22.24 ± 2.88 %) (n= 6 for quercetin, luteolin, urolithin A, n= 5 for resveratrol and apigenin, n= 3 for fisetin, n= 2 for flavone, 6-hydroxyflavone and 6-methoxyflavone; Figure 20).

A statistical significance for the effects of luteolin, urolithin A, apigenin, quercetin, fisetin, and resveratrol ($p= 0.001$) was calculated by one-way ANOVA with Tukey HSD test. The

statistical analysis revealed that the difference between the effect of luteolin and resveratrol was statistically significant as well (p= 0.008; Figure 20).

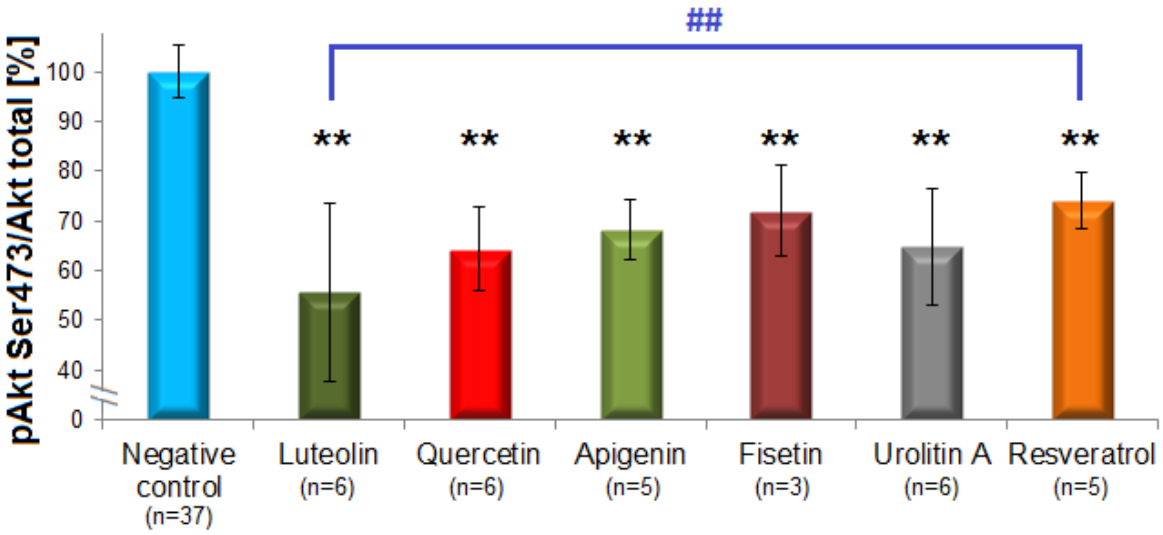


Figure 20: Effects of the polyphenolic compounds from different subclasses at a concentration of 10 µM on Akt-phosphorylation (pAkt Ser473) in EA.hy926 cells, determined by quantitative ELISA. The columns represent the means of the residual phosphorylation and standard deviations (S.D.) (in %). A statistically significant inhibition was determined for the following compounds (mean inhibition ± S.D.): luteolin (44.31 ± 17.95 %), urolithin A (35.28 ± 11.80 %), fisetin (28.09 ± 9.09 %), apigenin (31.79 ± 6.16 %), quercetin (35.71 ± 8.33 %), and resveratrol (26.04 ± 5.58 %) (n = 3-6), ** p< 0.01. The difference between the effects of luteolin and resveratrol was statistically significant as well, ## p< 0.01 (one-way ANOVA with Tukey HSD test).

2.3.3 Discussion: Secondary screening

The results from the secondary screening for the effects of polyphenols on the pAkt Ser473 are consistent with the outcome from the primary investigation. The inhibitory potential of the substances already found to be active (quercetin, apigenin, luteolin, resveratrol) was confirmed and compounds that were determined to lack inhibitory effect were only slightly active or inactive. Careful comparison of the results revealed that the values obtained in both approaches were very similar for quercetin (primary vs. secondary screening: 40.46 ± 4.83 % vs. 35.71 ± 8.33 %) and resveratrol (25.85 ± 5.72 % vs. 26.04 ± 5.58 %). On the contrary, differences between the effects established through both methods were observed for luteolin (29.96 ± 11.06 % vs. 44.31 ± 17.95 %) and apigenin (22.57 ± 10.30 % vs. 31.79 ± 6.16 %).

Possibly this difference is due to the fact that Western blot analysis is a complex procedure and multiple steps (transfer, stripping, and chemiluminescent detection) can contribute to variations in results. This technique was used for the primary screening and is generally considered as a semi-quantitative method [296], while ELISA employed for the secondary screening is a quantitative method [297]. For this reason only the results obtained from the secondary screening were considered in following investigations. Furthermore, the analysis revealed that the inhibitory effect of luteolin was statistically significantly stronger than the one of resveratrol (Figure 20). This finding might be important for a quantitative structure-activity-relationship study and a lead structure optimization because the compared compounds belong to different structural groups.

A literature search did not come across any information about any previously performed comprehensive screening for the effects of polyphenols on Akt in endothelial cells. However, some similar studies were found. Various flavonoids were investigated in adipocytes for their activities on multiple targets related to insulin-signaling, among which was Akt-kinase [298]. In this study including 24 flavonoids, it was shown that luteolin and kaempferol statistically significantly reduced the activity of Akt in comparison to the untreated controls. In addition, quercetin, fisetin and apigenin tended to decrease Akt-activity, although their effects were not statistically significant. The authors proposed the insulin receptor and PI3K as direct targets of flavonoids. Among the tested compounds luteolin inhibited the phosphorylation of the insulin receptor β-subunit statistically significantly. This is generally in accordance with the outcome of the present investigation showing that some flavonoids are capable to inhibit Akt and that luteolin displayed the highest activity. The scope of the present study in regard to Akt was broader, including representatives from additional chemical classes as stilbenoids, urolithins, and phenolic acids, and investigating the effects of methylation and glycosylation.

In another study employing macrophages the effects of stilbenoids and their semi-synthetic derivatives on the PI3K/Akt-pathway were examined [299]. The results pointed out that piceatannol, monomethylpinosylvin and pinosylvin were the most potent inhibitors of Akt-phosphorylation. Synthetic ethers of pynosilvin (i.e. monoethyl, monopropyl, monoallyl) caused weak or no inhibition on the phosphorylation on Akt, indicating that probably space-demanding residues are not favorable for the activity. Similarly to the present results 10 µM resveratrol exhibited clear activity, while its dimethylated derivative pterostilbene was not active at the same concentration.

As Akt is considered as a major downstream effector of PI3K [300], works investigating the relationships between flavonoid structure and the inhibition of this kinase were also studied. Agullo et al. investigated the effects of 14 flavonoids from different groups on the enzymatic activities of purified PI3K *in vitro* [301]. The most active compounds were myricetin, luteolin, apigenin, quercetin, and fisetin, which corresponds well with the data obtained in the present secondary screening (B-2.3.2). Likewise, flavan-3-ols, flavanones, isofalvones and morin were shown to be inactive. This might suggest that PI3K is a major molecular target of the investigated polyphenols. Some differences, as for instance stronger effects of luteolin compared to myricetin in the cell-based model, can be explained with a chemical stability as myricetin is known to be very unstable in cell culture media [272]. An additional reason can be the complex regulation of Akt, in which additional upstreaming pathways might be involved along with PI3K. Cell-membrane permeability of the tested substances is also playing a role in cell-based models. Another study investigating the inhibitory activity of flavonoids against specific class I isoforms of PI3K reported similar results [302]. In addition, a recent study linked the inhibitory potential of flavonoids to their binding with the pleckstrin homology domain of phosphoinositide-dependent kinase 1 (PDK1), which was also described to be an upstream regulator of Akt [303]. Thus, the literature search gave an overview for relevant upstream targets which might mediate the observed activities of polyphenols in the present study.

2.3.4 Conclusion: Secondary screening

The secondary screening confirmed the outcome of the primary screening and re-examined the effects of the active polyphenols quantitatively and found further molecules possessing activities higher than 20 % which might serve as a base for structure-activity relationship studies (SAR).

Four compounds that to our best knowledge were not previously described to inhibit the phosphorylation of Akt were detected during screening: 6-hydroxyflavone, 6-methoxyflavone, 7-methoxyflavone, and pinostilbene.

2.4 Semi-quantitative structure-activity-relationship (SAR)

2.4.1 Structure-activity-relationship: Key features

Based on the results of the comprehensive screening, a first assumption for the structural differences of polyphenols' molecules that might influence their inhibitory activities on the phosphorylation of Akt at Ser473 was made (Figure 21).

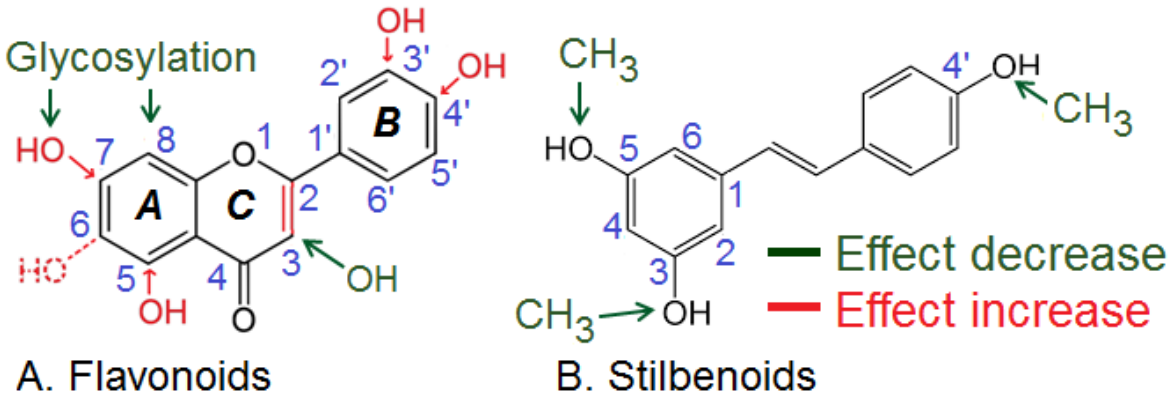


Figure 21: Proposed semi-quantitative structure-activity-relationships for flavones/ flavon-3-ols (A) and stilbenoids (B). Structural changes characterized leading to a decrease in the inhibition of Akt-phosphorylation are marked in green color. The changes shown in red were determined as likely increasing to the inhibitory potential of polyphenolic compounds.

Several sites of the polyphenols' molecules appeared to be important for the inhibition of Akt-phosphorylation by the investigated compounds. The presence of double bond C2=C3 (ring C) seemed to be essential for the inhibitory activity of flavones and flavone-3-ols. This assumption was drawn from a comparison between the inhibitions caused by quercetin (35.71 ± 8.33 %) vs. taxifolin (8.69 %), and apigenin (31.79 ± 6.16 %) vs. naringenin (–7.32 %). In addition, it was observed that a hydroxylation at C3 (ring C) reduced the inhibition potential of flavones/flavon-3-ols. Evidence for that were the differences of the following effects: luteolin (44.31 ± 17.95 %) vs. quercetin (35.71 ± 8.33 %), and apigenin (31.79 ± 6.16 %) vs. kaempferol (17.02 ± 11.81 %). Methylation of the hydroxyl group (C3) seemed to partially prevent this reduction in the inhibitory activity. This was concluded by a comparison between the effects of 3-methoxyflavone (12.85 %) vs. 3-hydroxyflavone (6.34 ± 4.22 %). Compounds characterized by a single hydroxylation or methylation at C6-position (Ring A) also possessed distinguishable inhibitory activities (> 20 %: 6-hydroxyflavone and 6-methoxyflavone). On the other hand, the most active flavones (luteolin, apigenin) were hydroxylated at C5- and C7- position of the Ring A, which might suggest that these structural features were important for the inhibitory potential. Since the Ring B in the molecules of the most active flavones/ flavone-3-ols (e.g., luteolin, fisetin, apigenin, quercetin) was hydroxylated, the OH groups (Ring B) most likely contributed to the inhibitory effects as well. The presence of a meta- and a para- OH groups seemed to be optimal for the activity [evidence: luteolin (m, p-OH)> apigenin (p-OH)> chrysin (Ø OH); 3,4'-dihydroxyflavone (p-OH)> 3-hydroxyflavone (Ø OH)]. Glycosylation of polyphenols occurs commonly in the nature. The investigated glycosides of active compounds showed no effects on Akt-phosphorylation. It seemed that this modification abolished the inhibitory potential. This was evident by comparison of the activities of apigenin (31.79 ± 6.16 %) vs. vitexin (–8.51 %), baicalein (18.18 %) vs. baicalin (–10.85 %) and wogonoside (–5.02 %).

For the subclass of stilbenoids, the presence of the three free OH groups on the 3-,4'-, 5-positions was probably optimal for the inhibitory effects. Methylation of these groups seemed to reduce or even to completely eliminate the inhibitory effects (resveratrol (26.04 ± 5.58 %) > pinostilbene (19.34 ± 14.45 %) > pterostilbene (–5.96 ± 1.59 %) ≈ 3,4',5-trimethoxy-trans-stilbene (0.93 ± 9.99 %)).

The conclusions about the relationship between the structural features and their influence on the inhibitory potential of polyphenols, along with the respective evidence are summarized in the Table 4.

Table 4.: Summary of the semi-quantitative structure-activity-relationships of flavones/ flavonoles and stilbenoids (3-MS: 3,4',5-trimethoxy-trans-stilbene):

№	Structural features	Possible effect	Evidence
Flavones/Flavon-3-ols			
1	C2=C3 double bound (Ring C)	**Essential**	Quercetin/Taxifolin; Apigenin/Naringenin
2	OH-groups (Ring B) *(m-, p-)*	**Contribution**	Luteolin (m, p) > Apigenin (p) > Chrysin (Ø)
3	3-p. (Ring C): hydroxylation	**Reduction**	Luteolin/Quercetin; Apigenin/Kaempferol
4	Glycosylation	**Abolishment**	Apigenin/Vitexin; Baicalein/Baicalin
Stilbenoids			
1	Three free OH-groups	**Optimal**	Resveratrol > Pinostilben > Pterostilben ≈ 3-MS
2	Methylation of OH-groups	**Abolishment**	

Regarding the subclass of urolithins (see also B-2.6), although its representatives were structurally related, clear differences in their inhibitory effects were observed (Figure 22).

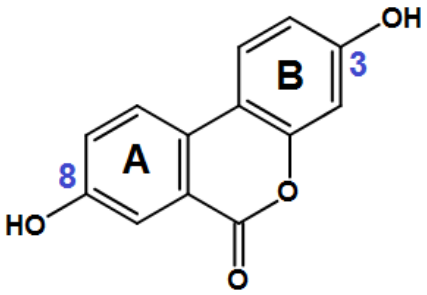


Figure 22: Urolithin A with OH-groups at C3 and C8 was the only active representative among the group of urolithins regarding pAkt-inhibition. Further hydroxylation at C4 or C9 as well as lack of OH-group at C8 was not favorable for the activity.

It seemed that two OH-groups at the C3 and C8 positions of the molecule and lack of further substituents were important for the activity. Thus, only minor changes, such as an addition or elimination of a hydroxyl group were responsible for a remarkable change in the inhibition. Similar observation was valid for flavonols: active quercetin and slightly active morin – analogs, differing only by the position of one phenolic OH-group in the B-ring.

All these assumptions were considered for the creation of a pharmacophore-based model (see B-2.5).

2.4.2 Discusion: SAR

Many studies investigated the relationship between polyphenol structure characteristics and molecular effects contributing to advantageous impact of these compounds on human health. For instance, assumptions were made for antioxidant activity [304], various enzyme inhibition [305], and cytotoxicity [306].

The results from the present screening involving multiple polyphenols revealed some differences in the activities of compounds on Akt-phosphorylation, which might be related to particular structural characteristics. Generally, most active compounds belonged to the subclass of flavones, followed by flavonols. For these two groups the structural hallmarks were double bound between C2 and C3 of the ring C, preferable lack of substitution at the C3 of the ring C, and 3',4'-catechol group in the ring B. The most active compounds were those with unsubstituted OH-groups (no methylation or glycosylation) which indicated that the formation of hydrogen bounds between the scaffold and hypothesized receptor molecule was favorable. The double bound C2=C3 was required for the co-planarity of the heteroring C. Such molecular geometry may facilitate a docking to a binding pocket of a protein.

Other studies investigating the structure-activity-relationship between polyphenols and PI3K also reported that the presence of C2=C3 double bound and ortho-catechol group on the ring B are important structural requirements for the inhibitory activity [301, 302]. A comparison between these studies and the present screening followed by SAR revealed similar outcomes regarding active and inactive compounds and defined features important for the inhibitory potential. This suggests that PI3K might be the major target of the investigated polyphenols responsible for the observed effects on Akt-phosphorylation.

Phosphorylation status of Akt was reported to be dependent on the oxidative stress levels [307]. Therefore, the own assumptions about the structure-activity-relationship (SAR) regarding Akt-phosphorylation was compared the antioxidant activity of polyphenols. In this way it was examined if the antioxidant properties of polyphenols might be related to the observed results concerning Akt. According to the Bors' criteria [308] C2=C3 double bound is beneficial for the antioxidant activity of flanonoids, as it was responsible for the electron delocalization over all tree rings of the system and thus contributed to the radical stabilization. In addition, ortho-catechol structure in the ring B was considered important as it assured the stability of flavonoid phenoxyl radical by hydrogen bound. Also, the presence of 3-OH group (ring C) is beneficial for the activity (Table 5).

Akt-phosphorylation inhibition and the antioxidant properties differed from each other as the C-ring OH-group is favorable for radical scavenging activity [309], but negatively influenced the inhibitory potential of poylphenols on pAkt. An additional difference was the effect of glycosylation. It decreased the antioxidant activity compared to the aglycones [310], but seemed to abolish and even reversed the inhibitory activity regarding pAkt in the present study (Table 5).

Table 5.: Comparison between the assumptions for structure-activity-relationship regarding inhibitory effects on Akt-phosphorylation (pAkt) based on the present results and the antioxidant properties of polyphenols [309]. Effects of functional groups that differ between both assumptions are shown in red.

Functional characteristic	**Inhibition of pAkt**	**Antioxidant activity**
Double bound (C2=C3)	Increase	Increase
OH-group in ring A	Increase	Increase
OH-group in ring B	Increase	Increase
OH-group in ring C (3-OH)	Decrease	Increase
Glycosyl group	Abolish/Reverse	Decrease
O-Methyl group	Decrease	Decrease

2.4.3 Conclusion: SAR

The semi-quantitative SAR described in the present study assumed which functional groups were important for the inhibitory activity of polyphenols on Akt-phosphorylation. It was hypothesized that PI3K-inhibition, but not solely the antioxidant properties of those polyphenolic compounds might play a major role for their effects on the Akt-kinase.

2.5 Pharmacophore-based model for Akt-inhibition

The pAkt inhibitory activities of investigated polyphenols (see B-2.3) belonged to a relative narrow range (0 – 45 % inhibition) and for this reason only structural features of the most active four substances were used as a base for establishment of a pharmacophore-based model. The *in silico* study's workflow was comprised of compounds selection, flexible alignments, pharmacophore-based model description, validation, virtual screening, and multifactorial analysis of the hits.

2.5.1 Compounds selection

The first step for the creation of a predictive pharmacophore-based computer model was the selection of active substances to be used as an input. The criterion for inclusion was inhibitory activity stronger than 20 %. The most active representatives from four different structural subclasses were: luteolin (44.31 % inhibition), quercetin (35.71 %), resveratrol (26.04 %), and urolithin A (35.28 %) (B-2.3.2). At first glance, these molecules shared some common features: all of them had relative planar geometry and were conjugated systems (Figure 23).

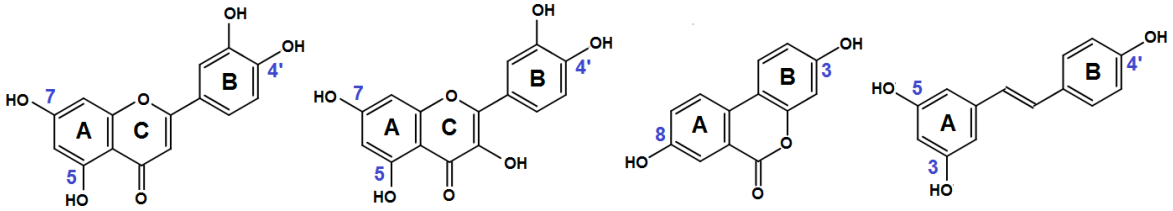


Figure 23: Input substances for creation of pharmacophore-based *in silico* model and their inhibitory potentials on Akt-phosphorylation (in %).

2.5.2 Flexible alignments

In order to uncover similar structural characteristics the selected molecules were superposed, using the tool "Flexible Alignments" available in the Molecular Operating Environment program (MOE) [311]. This methodology was developed by Labute et al.

[312] and is widely used for a pharmacophore elucidation. A force field suitable for small molecules (MMFF9x) was selected and was used for all further actions in the MOE.

A collection of alignments was generated and evaluated using a special scoring quantifying the quality of the overlay [313] (for details refer to C-2.5). The best five molecular superpositions were investigated as a base for the creation of a pharmacophore model. Besides the scoring, the number of common structural features defined (see the next point) indicated that the first alignment (Figure 24) was superior.

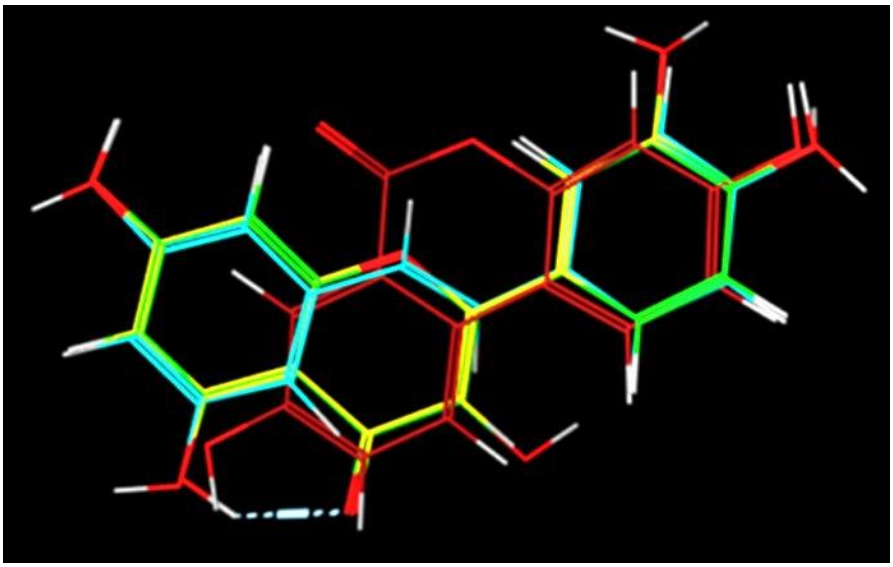


Figure 24: The best-scored flexible alignment generated by the Molecular Operating Environment (MOE) program, based on the molecules of luteolin (yellow), quercetin (green), resveratrol (blue), and urolithin A (red), which were the most active representatives from the chemical subclasses they belong. The overlay indicated that all four compounds shared multiple common structural characteristics and molecular geometry.

In addition, distances between of the hydroxyl groups of the polyphenols were measured:

- Luteolin (5- to 4'- OH): 10.78 Å
- Quercetin (5- to 4'-OH): 10.80 Å
- Urolithin A (3 to 8'-OH): 9.77 Å
- Resveratrol (3 to 4'-OH): 10.77 Å

2.5.3 Pharmacophore-based model establishment

Using the tool “Pharmacophore query” from the MOE enabled a description of common characteristics for all compounds included in the flexible alignment which might be important for the activity. Initially, seven features were defined to be shared (100 %) between all four input molecules (Table 6).

Table 6.: List of shared (100 %) features between the aligned molecules of quercetin, luteolin, resveratrol, and urolithin A defined by the “Pharmacophore query” tool from the MOE program:

Abbreviation	Description	Structural elements
Don&Acc 1	Aligned OH-groups: hydrogen-bond donor & hydrogen-bond acceptor	4’-OH of luteolin and quercetin, 4’-OH of resveratrol, 3-OH of urolithin A
Don&Acc 2	Aligned OH-groups: hydrogen-bond donor & hydrogen-bond acceptor	5-OH of luteolin and quercetin, 3-OH of resveratrol, 8-OH of urolithin A
Aro|Hyd 1	Aligned aromatic rings	B-rings of luteolin and quercetin, monohydroxylated B-ring of resveratrol and B-ring of urolithin A
PiN 1	Planar projection of the mentioned **Aro|Hyd 1**	above the alignment plane
PiN 2	Planar projection of the mentioned **Aro|Hyd 1**	beneath the alignment plane
Aro|Hyd 2	Aligned hydrophobic regions	Pyrone rings of quercetin, luteolin (ring C) and benzene A-ring of urolithin A, along with the double bond of resveratrol
Acc2	Abstract corresponding projected point of an acceptor on the receptor molecule	Defined by: -7-OH-groups from luteolin and quercetin, -5-OH-group from resveratrol -Carbonyl group of urolithin A

Those features were graphically displayed (Figure 25).

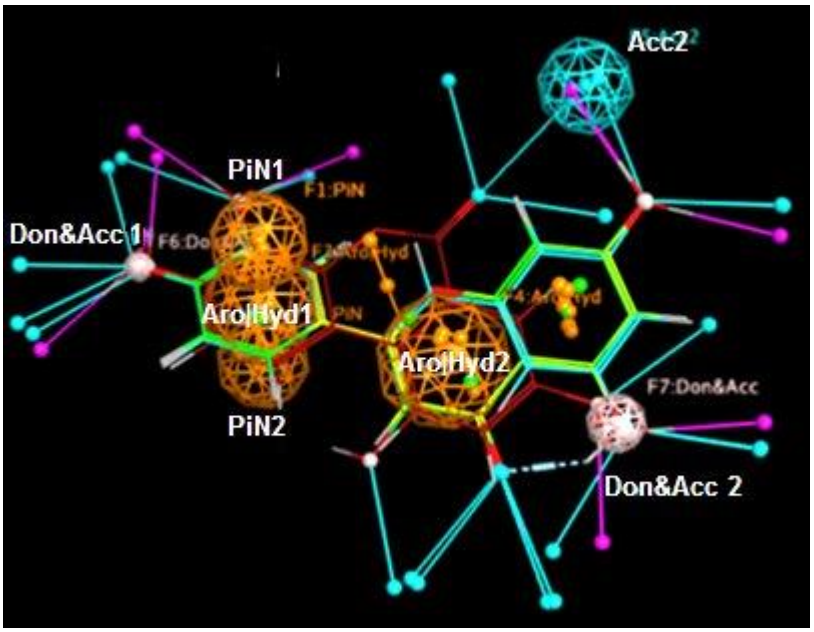


Figure 25: Representation of the pharmacophore-based model of the most active polyphenols from four subclasses, inhibiting Akt-phosphorylation, generated by the MOE. Totally seven structural features were defined as common for all molecules included: Don&Acc 1, 2 (hydrogen bond donor and acceptor), Aro|Hyd 1, 2 (aromatic or hydrophobic region), PiN 1, 2 (aromatic ring projection), and Acc2 (abstract acceptor projection).

2.5.4 Validation and improvement of the model

To validate the established model a database consisting of 44 compounds with conformers of all molecules included in the *in vitro* screening process (B-2.3) for inhibitory potential on Akt-phosphorylation (active and inactive) was generated. Again, compounds possessing inhibitory potential around 20 % or more were considered active. This database was screened in order to validate that the created *in silico* model was capable to recognize only active molecules in the process of virtual screening. As many of the compounds had similar structures, the pharmacophore query search found some molecules without activities according to the *in vitro* results. This demanded further optimization of the model. Initially the radiuses of the spheres describing common features (Don&Acc 1, Don&Acc 2, PiN 1, PiN 2, Table 6) were varied and thereby many inactive compounds were sorted out. Further two different strategies were used to improve the model: the exclusive (B-2.5.4.1) and the planar (B-2.5.4.2) pharmacophore-based models.

2.5.4.1 Exclusive pharmacophore-based model

As some active compounds differed structurally from the inactive ones only by a substituent or by the presence/absence of a hydroxyl group (urolithins C and D, morin), these residues were extra excluded by the "volume exclusion" option of the MOE. Bulky groups were not favorable for the activity and were additionally excluded as well (gallic acid from EGCG and (–)-quinic acid from chlorogenic acid, Figure 26).

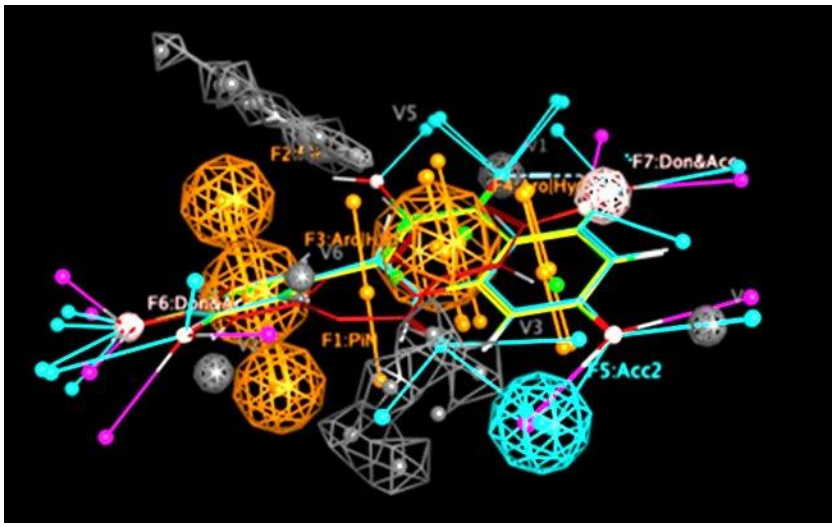


Figure 26: Exclusive pharmacophore-based model (MOE). In gray are shown the volumes of bulky groups and hydroxyl groups which were excluded in order to eliminate the respective inactive molecules ((-)-EGCG, chlorogenic acid, urolithin C, D, morin) during the validation process.

2.5.4.2 Planar pharmacophore-based model

This strategy was based on the observation that all four input molecules possessed relatively planar geometry and that probably the hypothetic target might have only a narrow binding pocket. For this reason the volumes above and beneath the superposed molecules were excluded. The distance in between was approximately 4.2 Å ("excluded volume"). This limited the appearance of inactive molecules with large substituents ((-)-EGCG, chlorogenic acid, glycosides) in the output during the validation. Morin could not be eliminated and its additional hydroxyl group was specially excluded from the model (V4, V6). The planar pharmacophore-based model is represented on the Figure 27.

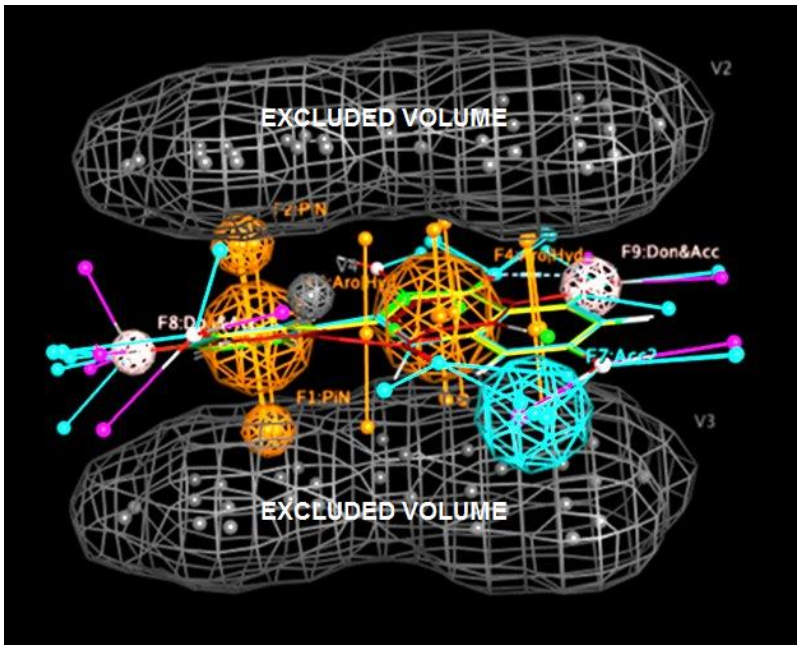


Figure 27: Planar pharmacophore-based model (MOE) based on the assumption that the active compounds should have a planar geometry based on the structures of the input compounds. The volumes above and beneath the superposed molecules were excluded (excluded volume). This model successfully limited the appearance of inactive molecules with large substituents during the validation process.

Thus, the pharmacophore-based models were optimized and after the validation process only the most active five molecules (luteolin, apigenin, quercetin, resveratrol, urolithin A) along with kaempferol and myricetin (activity around 20 %) were recognized.

2.5.5 Virtual screening

Both versions of the *in silico* model were used to perform virtual screening of a large dataset of compounds from five databases selected and filtered by defined criteria (see C-2.5). Only molecules that were purchasable were included. The databases were obtained from the following sources: Enamine (HTS, Advanced Structures) [314], Life Chemicals [315], eMolecules [316], Sweatlead [317], and DrugBank [318]. Prior to the screening, a creation of 3D conformers of molecules was performed with the MOE (see C-2.5).

Approximately 8 million compounds were subjected to a virtual screening. Around 100 hits were detected. The codes, chemical structures of the hits are listed in the Supplement, Section D-3, Virtual screening. The small number of substances recognized by the model indicated that it possessed high selectivity.

2.5.6 Post-screening-analysis

To predict the activity and relevance of the hits for further investigations, important structural features, conformations, physicochemical properties and similarity to already investigated molecules from the *in vitro* screening study were considered.

According to the primary semi-quantitative SAR (B-2.4.1) glycosides possessed no *in vitro* inhibitory activity at all. For this reason all hits containing sugar-residues were excluded from the analysis. The exclusive pharmacophore-based model recognized some conformers from catechins and they were not studied further as well. Molecules that were quite similar to some of the active ones (i.e. additional hydroxyl or methyl group) were also not of interest.

A further step in the post-screening-analysis was the calculation of descriptors defining the relevance of substances for *in vitro* analysis in cell-based assays. These included acid-base properties (pK_a- and pK_b-values) of the compounds and respectively their charges (protonated/deprotonated form) under cell culture conditions (pH= 7.4 – 7.6), along with their water solubility (partition coefficient o/w: $LogD_{7.4}$). These parameters were calculated using the MoKa software [319]; (C-2.5). Possible tautomerization was also predicted and chiral centers were considered.

The pK_a- and LogD-values and charges were calculated for the input compounds in order to serve as a base for comparison with those properties of the hits (Table 7).

Table 7.: Predicted strongest acidic pK_a-, LogD-, LogP-values, and charges of the input compounds by MoKa software; qp – quality of prediction, qp= 0 corresponds to a good prediction. The bigger is the absolute value of qp, the lower is the quality of predictions.

Substance	$LogD_{7.4}$	$LogP_{o/w}$	Strongest acidic pK_a	predicted charge at pH 7.4	qp
Quercetin	–0.9	2.0	4.48	(–)	0.00
Resveratrol	1.8	2.9	6.20	(–)	0.00
Urolithin A	3.2	3.2	8.29	(neutral)	0.00
Luteolin	0.5	2.4	5.34	(–)	0.00

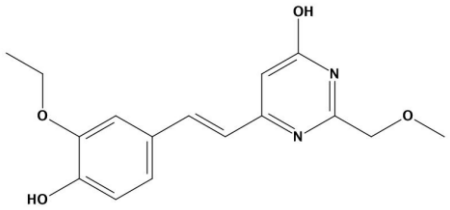
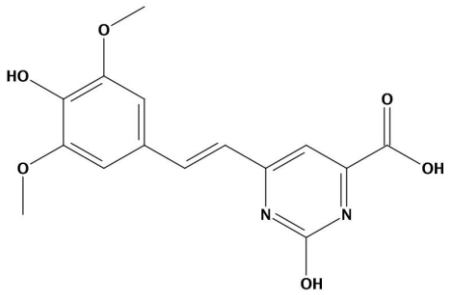
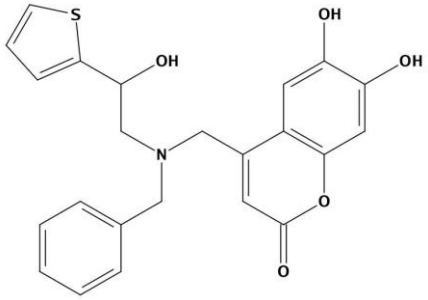
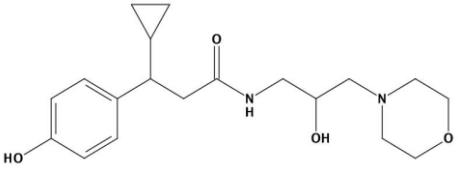
Accordingly, these parameters were also calculated for the hits. Hits having predominantly positive charged species or high values of $LogD_{7.4}$ (indicating poor solubility) were excluded from further analysis.

Lastly, a systematic conformational search was performed for short-listed compounds. All 3D-conformers generated were studied for their eligibility to satisfy the pharmacophore models and whether the matches were energetically favorable. Molecular conformations, having relative conformer energy dE < 3 kcal/mol were considered stable.

2.5.7 Summarized results (*in silico*)

After the post-screening analysis of the hits obtained from the virtual screening (B-2.5.5), four compounds with possibly suitable properties were chosen for an experimental "proof of concept", respectively *in vitro* validation of the predictive model (Table 8).

Table 8.: List of hits, along with their vendor codes (from Enamine store), structures, pK_a-, LogD-values, and relative conformational energy dE (regarded to the most stable conformer) selected for the *in vitro* validation of the model; qp – quality of pK_a-value predictions, qp= 0 corresponds to a good prediction. The bigger is the absolute value of qp, the lower is the quality of predictions (MoKa):

No	Code	Chemical structure of the hits	LogD	Strongest acidic pK_a-value, predicted charge	Energetically favourable conformation (dE) [kcal/mol]
1	Z2752916184		1.3	6.84 (–) qp= –0.28	2.62
2	Z1567937963		–2.0	2.78 (–) qp= –2.61	0.00
3	Z1621797376		2.6	8.07 (neutral) qp= 0.00	2.31
4	Z2329004358		0.5	9.79 (neutral) qp= –0.02	7.78

2.5.8 Discussion: *In silico* analysis

After model generation and validation four molecules were suggested to have a potential Akt-inhibitory activity. The selected compounds had physicochemical characteristics close to those of the input substances, according to the predicted values (MoKa), with some exceptions. The results obtained from the program for pKa-values were different than the experimentally established ones found in the literature. The strongest acidic OH-groups of resveratrol, luteolin, and quercetin were predicted to have pK_a-values of 6.20, 5.34, and 4.48, while the experimentally determined values were 8.8 [320], 6.9 [321], and 7.1 [322]. These differences might be due to the training set and algorithm used by MoKa [323]. As all predicted pK_a-values were lower than the experimentally established, a systematic error could be assumed. Thus, charges of the hits were not expected to differ much from the input substances' ones at pH 7.5, except for Z1567937963 (compound 2, Table 8) which was expected to be strongly deprotonated and negatively charged, because of its carboxyl group. This might compromise the cell-membrane permeability of this compound in *in vitro* experiments.

The energies of the conformers of the hits matching the pharmacophore model were considered. Compound 2 (Table 8) had energetically very favorable conformations which satisfied the pharmacophore-based model and molecular structure close to this of resveratrol. Compound 4 was to a big extent non-charged, but the relative energy of its model-matching conformations was probably too high, and respectively required conformations too unstable. Anyway, it was considered as a compound with a different structure from the input compounds to test.

Compound 1 was a stilbene analogue, having predicted pK_a-value similar to this of resveratrol and stable model-matching conformers. A very important factor to consider was an existing tautomerization defined by MoKa (Supplement, Section D-3). Compound 3 had a hydroxylated benzo-coumarin fragment, similar to urolithin A. Its conformers matched both pharmacophore models. Matches of the exclusive model were with more favorable energies than those matching the planar model. This might help to distinguish if the active compounds should necessarily have a planar structure.

The multifactorial analysis indicated that among the selected compounds there was no one which perfectly fulfilled all parameters (charge, conformer stability, absence of tautomerization and chiral centers) of criteria for relevance and applicability to *in vitro* studies.

2.5.9 Limitations of the study

The strategy used in the pharmacophore-based modeling assumed that the input compounds had similar mechanism of action and their shared structural features were involved in it. In addition it was supposed that the mode-of-action is ligand-target based.

Despite the fact that the direct target of polyphenols affecting Akt-phosphorylation was unknown, it was conceivable that the active compounds might interact with upstream pathways of Akt-kinase. As discussed under B-2.4.2 it was suggested that polyphenols interacted directly with PI3K. Indeed, in the literature was reported that quercetin [262], resveratrol [324], luteolin [325], and urolithin A [326] are capable to inhibit PI3K, indicating that the selected polyphenols might have similar mode of action regarding inhibition of Akt-phosphorylation. Thus, the assumption about the planar model (B-2.5.4.2) might be correct.

An additional limitation was that the model might have been too restrictive, as it did not recognize some compounds that possess activity greater than 20 % (B-2.3.2: i.e. 6-hydroxyflavone, 6-methoxyflavone) in the validation process. However, the model was validated and optimized for virtual screening and based on the hits found and an *in vitro* validation of their activity it could be expanded and further improved. Furthermore, the established pharmacophore-based model might be connected to the binding site of PI3K or other relevant up-stream kinases of Akt and might be re-examined and advanced (B-2.5.11).

2.5.10 Proof of concept – results

In order to verify that the *in silico* model is able to predict the inhibitory activity on Akt-phosphorylation of novel compounds, the selected hits listed in Table 8 (Compounds 1–4) were tested *in vitro* in the experimental set-up used for the initial screening of polyphenolic compounds (C-2.4.1.4) under the same conditions.

The results from independent experiments (n= 5 – 10) indicated that all four tested compounds did not exhibit any significant effects on the phosphorylation of Akt in endothelial cells. After treatment with each substance, the phosphorylation of Akt remained unchanged compared to vehicle-treated negative controls (Figure 28).

Detailed information about the descriptive statistics for the effects of the virtual screening hits on Akt-phosphorylation can be found in the Supplement, Section D-3. In addition, the phosphorylation status of Akt on Thr308 position remained likewise unaffected after treatment (data not shown).

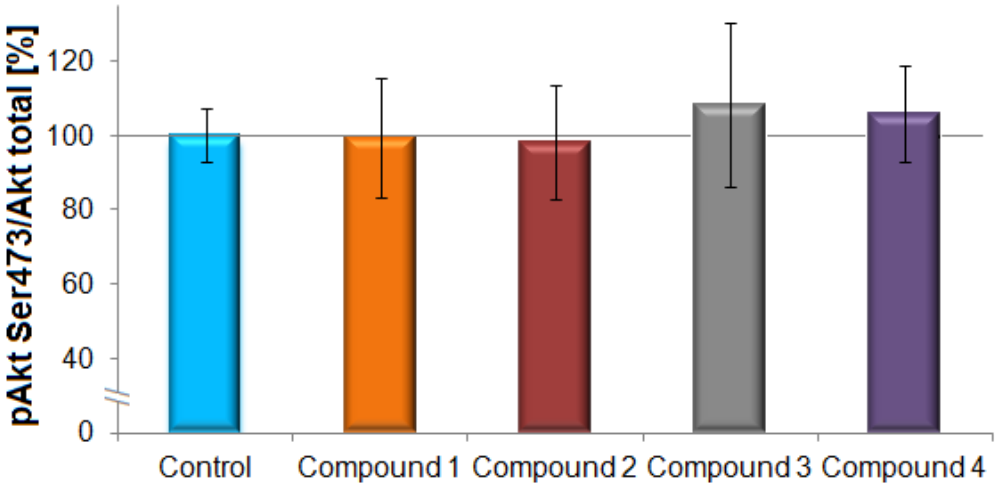


Figure 28: **Effects of the virtual screening hits (Compounds 1–4) on Akt-phosphorylation in endothelial cells. Columns represent mean values, error bars represent standard deviations. The investigated compounds induced no statistically significant changes on the phosphorylation status of Akt at Ser473 under the same conditions used for screening of polyphenolic compounds (n= 5 – 10, one-way ANOVA/Tukey HSD test). Thus, the *in vitro* validation was not able to confirm the proposed *in silico* concept.**

2.5.11 Proof of concept – discussion

The *in vitro* investigation of the selected virtual screening hits uncovered that none of them were active modulators of Akt. Several plausible explanations for this outcome exist. Even after the multifactorial analysis none of the selected substances was an ideal match (B-2.5.8). In addition, four substances might be insufficient to cover the structural diversity and exact hit parameters need to be present in order for a molecule to be active. For instance, Compound 1 and Compound 2 exhibited structural similarity to resveratrol. However, both compounds have additional ether groups (Table 8) which are bulky and can spatially hinder interaction with a given molecular target (e.g. a binding pocket). No large residues were present in the structures for the other input molecules as well (B-2.5.1). Virtual screening is limited to defined structures available in the databases employed. Certain flexibility can be achieved, if the compounds to be tested can be synthesized following given structural criteria provided by the pharmacophore model and according to a defined algorithm.

Data for the input compounds shows they might directly interact with PI3K (B-2.5.9). Therefore, it was assumed that the pharmacophore model might match some structural features from the binding pocket of this kinase. This might suggest reliability of the *in silico* model, if confirmed. The pharmacophore model was fitted to the ATP-binding pocket in the crystal structure of PI3K [327]. Indeed, it was a good blend between some

features from the pharmacophore model and the protein structure (Figure 29 A). The hydrophobic feature Aro|Hyd 1 (C-ring) (B-2.5.3) corresponded well to methionine residues from both sites with approximate distances of 3.90 and 5.30 Å. A possible interaction between sulfur atom from methionine and an aromatic ring's π-system has been described for distance < 6 Å [328]. Aro|Hyd 2 (B-ring) complies with the hydrophobic isoleucine rest. PiN 1 and 2 could be also considered relevant because of the planarity of the binding pocket. Don&Acc 1 (OH-group) interacts with an aspartate carboxyl group. In addition, excluded volumes (because of inactive compounds, such as morin; V4, V6) indicate that OH-groups there might not be favorable for the activity because of the proximity of hydrophobic isoleucine rests (distance around 2Å). Don&Acc 2 does not seem to interact with any of the amino acids from the ATP-binding pocket of PI3K. On the contrary, a feature (Acc) based on the carbonyl functions of quercetin and luteolin (but then not fully considering determinants of urolithin A and resveratrol) can also be considered relevant as it potentially can interact with a NH_2-group of valine. It seems that the pharmacophore model matches well the shape, the hydrophilic (purple) and hydrophobic (green) regions of the PI3K's binding pocket surface (Figure 29 B).

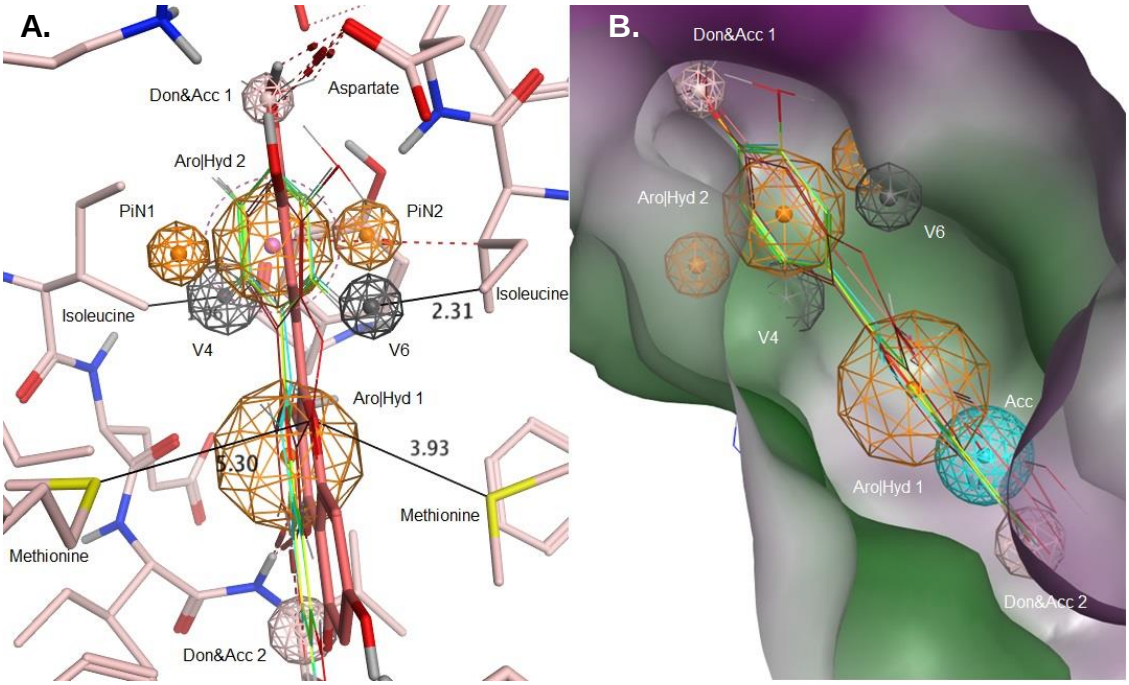


Figure 29: Fitting of the pharmacophore model into the PI3K binding pocket, where quercetin had been docked (Walker et al. [327]). A: The interactions giving reliability of the model are: OH-group (Don&Acc 1) with aspartate carboxyl group; Pyrone ring (Aro|Hyd 1) with two methionine residues (yellow; distance: 5.30 and 3.90 Å); B-benzene ring (Aro|Hyd 2) with isoleucine rest; Excluded volumes (V4, V6) for OH-groups – vicinity to hydrophobic isoleucine residues: special hindrance. B: Pharmacophore model matches well the shape and polarity (hydrophilic (purple) and hydrophobic (green) regions) of the PI3K's binding pocket surface.

Despite the inactivity of the selected hits, it cannot be concluded that the pharmacophore model is not reliable. Conversely, the proposed *in silico* model complies well with the protein structure of a kinase upstream of Akt – PI3K. The model can be further improved and testing of further compounds *in vitro* is needed to verify its relevance.

2.6 Investigation of the effect of bio-activation of polyphenols

2.6.1 Background: Bio-activation of polyphenols

Recently the impact of intestinal microbiota on the human health is of increasing interest and object of extensive research activities [329, 330]. Various clinical and epidemiological studies indicated an inverse relationship between the intact gut microflora and T2DM and its related metabolic syndrome [331-333]. Among the variety of mechanisms proposed to be responsible for the health benefits the microbial transformation of nutrient precursors into bioactive metabolites plays a significant role. Such metabolites are for example short chain fatty acids and trimethylamine N-oxide [334]. An important group of nutrients are polyphenols. Evidence exists that some polyphenolic compounds can be likewise metabolized and thus can undergo bio-activation. In particular, gut-microbiota-generated metabolites of lignans were shown to reduce the risk of T2DM [335].

In addition to lignans, further polyphenolic compounds described to undergo bio-activation are ellagotanins and ellagic acid which can be bio-transformed by intestinal microbiota into urolithins, which were shown to exhibit various pharmacological effects [330, 336]. Urolithins exhibit stronger biological activities than ellagic acid and are up to 80-fold more bioavailable in human plasma than their precursor [337].

Catechin metabolites M1 and M2 were shown to occur in blood plasma of individuals after intake of French maritime pine bark extract [165]. *Eggerthella lenta* and *Flavonifractor plautii* bacterial strains were described to convert catechin and its oligomers to delta-(3,4-dihydroxy-phenyl)-gamma-valerolactone (M1) [338]. This compound was capable to statistically significantly inhibit inducible nitric oxide synthase (iNOS), while in contrast its precursor (+)-catechin had a minor effect [140]. In addition, another study revealed that bacterial metabolites M1 and M2 were more potent inhibitors of matrix metalloproteinases (MMP-I, MMP-II and MMP-IX) than (+)-catechin [180].

Thus, polyphenolic compounds may undergo bio-activation through metabolic conversion by intestinal microbiota.

2.6.2 Results: Effect of bio-activation

Polyphenolic compounds and their corresponding intestinal microbiota-generated metabolites were analyzed in parallel for their *in vitro* inhibitory potential on the Ser473 Akt-phosphorylation by ELISA (C-2.4.5). (+)-Catechin was compared with its microbial metabolites M1 and M2. (+)-Catechin caused a slight statistically non-significant increase of Akt-phosphorylation with 9.3 ± 5.76 % (n= 3; mean inhibition ± S.D.). While M1 showed no influence on Akt-phosphorylation (n= 1), its methylated form M2 tended to increase pAkt with 8.93 ± 8.67, although this effect was not statistically significant.

In contrast, there was a difference between the effects of ellagic acid and its metabolites. While ellagic acid (n= 3) had a little effect on Akt-phosphorylation (12.41 ± 4.27 %), urolithin A (n= 6) induced relatively strong and reproducible inhibition (34.72 ± 11.90 %, p= 0.001 **). The other urolithins (urolithin B, C, D) had no statistically significant inhibitory activity on Akt-phosphorylation (n= 1, Figure 30).

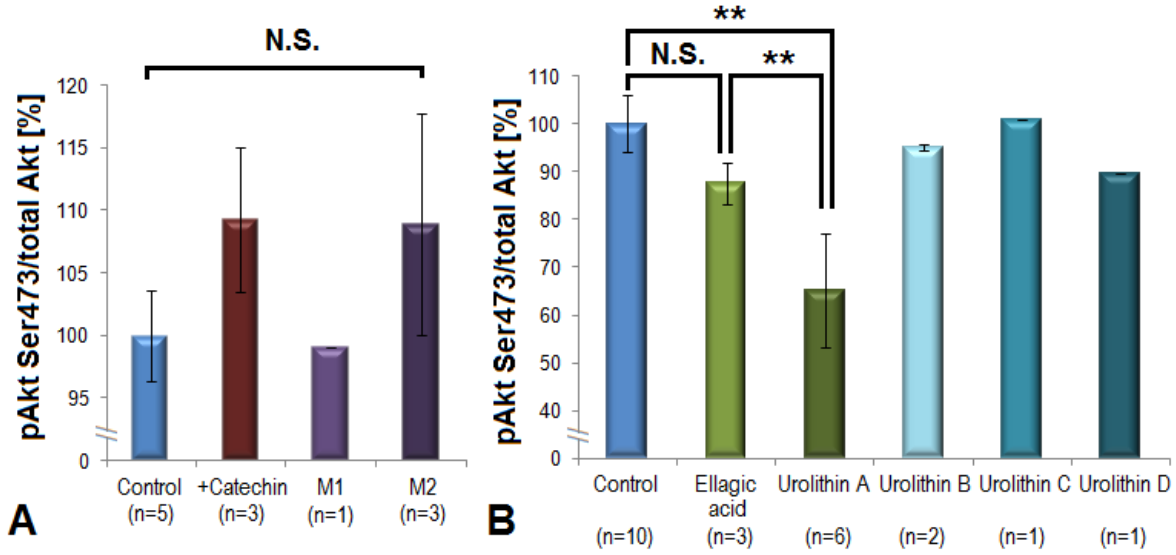


Figure 30: Investigation of the effect of bio-activation of polyphenols by intestinal bacteria. A. (+)-Catechin was compared with its microbiota-generated metabolites M1 and M2. (+)-Catechin and M2 caused non-statistically significant increase in Akt-phosphorylation, but M1 showed no activity. B. Ellagic acid influenced the phosphorylation of Akt only slightly (N.S., p= 0.1). In contrast, its intestinal bacteria metabolite urolithin A induced relatively strong and reproducible inhibition (34.72 ± 11.90 %) on Akt-phosphorylation (p= 0.001, mean ± standard deviation). The difference between the effects of ellagic acid and urolithin A was statistically significant (** p= 0.005, one-way ANOVA/ Tukey post-hoc test). The other urolithins showed only minor inhibitory effects (n= 3 – 6 for (+)-catechin, M2, ellagic acid, and urolithin A, n= 1– 2 for the other substances).**

2.6.3 Discussion: Effect of bio-activation

(+)-Catechin and its metabolites M1 and M2 were not active regarding Akt-phosphorylation in endothelial cells. Thus, no effect of bio-activation was observed.

A bio-activation of ellagic acid was shown for different targets such as heme peroxidases [339] and estrogen receptors [340]. Likewise, the present data suggested a bio-activation of ellagic acid by microbiota metabolism regarding the diabetes relevant target Akt-kinase in endothelial cells. Among the tested metabolites only urolithin A exhibited a clear inhibition of Akt-phosphorylation, in spite of the structural homology to the other urolithins. This suggests a specific effect of urolithin A.

The own results are consistent with a study showing that only urolithin A inhibited statistically significantly the phosphorylation of Akt compared to controls, while urolithin B and C did not cause any changes in bladder cancer cells [341].

2.7 "Metformin-like" effects of polyphenols

2.7.1 Background: "Metformin-like" effects of polyphenols

Metformin is used as first-line treatment of T2DM because of its cost-effectiveness and safety profile (see also A-1.6) [342]. Although it is widely used its molecular mechanism of action is not completely clarified. Along with its glucose-lowering activity, metformin was shown to possess further benefits for human health, such as cardio-protective [343], anti-obesity [344], anti-aging [345], and indirect antioxidant effects [346]. Additionally, metformin improved vascular endothelial dysfunction in T2DM patients [347]. Similar effects as those described for metformin were reported for polyphenols as well. Several studies aimed to find natural substances mimicking the activities of metformin [348-350]. Recently, Nyane et al. compared the effects and mode-of-action of the biguanidine drug and naringenin concluding that the polyphenol exhibits "metformin-like" activities [351].

2.7.2 Results: "Metformin-like" effects of polyphenols

The effects of metformin on Akt-kinase were investigated in endothelial cells (Ea.hy926) *in vitro*. Therapeutically relevant systemic plasma concentrations of the drug were reported from around 10 µM [352] up to 32 µM [353]. Therefore cells were treated with 10 µM metformin for 2.5 h. The phosphorylation of Akt was determined by Western blot (C-2.4.4).

The results of six independent experiments indicated that metformin did not influence Akt-phosphorylation statistically significantly ($p > 0.05$, $n = 6$, Student's t-test, two-tailed, paired). Practically no difference between untreated (100.00 ± 2.22 %) and treated cells (95.52 ± 5.72 %) was observed. In contrast, quercetin induced a clear inhibition of Akt-phosphorylation (Figure 31).

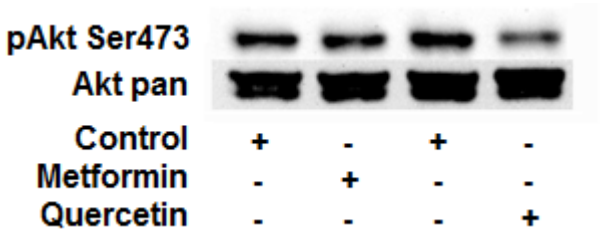


Figure 31: Representative Western blot comparing the effects of metformin and quercetin on the phosphorylation level of Akt at Ser473 (pAkt). Both substances were tested on Ea.hy926 endothelial cells at a concentration of 10 µM. In accordance with the previous results from the screening of polyphenols on Akt-phosphorylation quercetin caused a remarkable reduction of pAkt, while metformin induced no statistically significant changes.

As the biguanidine derivative did not influence Akt-phosphorylation in the present *in vitro* model, additional experiments with different treatment time points (i.e. 30 minutes) or with primary endothelial cells (HUVEC) were performed. However, metformin induced no statistically significant inhibition under any of the tested conditions (data not shown).

2.7.3 Discussion: "Metformin-like" effects of polyphenols

The influence of metformin on Akt-phosphorylation *in vitro* in endothelial cells revealed no inhibitory effects in the cell model used. The present work did not confirm the inhibitory potential of the anti-diabetic drug described in previous studies [18, 354].

However, preclinical data for metformin actions should be interpreted carefully. Multiple *in vitro* and *in vivo* studies were performed with supra-pharmacological concentrations which might yield misleading conclusions about the mode-of-action and effects of the drug [355]. Indeed, metformin between 10 and 60 mM was shown to exhibit anti-proliferative effects, mediated through an inhibition of Akt-phosphorylation [356, 357]. These concentrations were around 1000 times higher than the typical *in vivo* serum levels.

Othman et al. reported that 3 µM metformin protected kidney cells from insulin-induced DNA-damage through Akt-inhibition [18]. This finding could not be reproduced in endothelial cells model used in the present study, probably due to differences in the signaling between both cell lines and differences in the experimental design.

Another study claimed that 20 µM metformin antagonized short high-glucose-triggered Akt-activation in cultured human vascular endothelial cells isolated from saphenous veins [354]. In spite of varying the experimental conditions (cell type: HUVEC instead of Ea.hy926, metformin concentrations used: 10 and 20 µM, duration of treatment: from 10 min up to 3 h, stimulus used: growth factors, insulin, and 30 mmol/l glucose) the outcome of that study could not be confirmed in the own experimental setting.

Clinically important is the finding that metformin can be accumulated in erythrocytes [358]. Thus, a deep compartment for the drug may exist and an *in vivo* inhibitory effect on pAkt due to concentrations higher than detected in plasma cannot be completely ruled out.

Additional experiments performed indicated effects of both metformin and polyphenols on the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) (data not shown). Activation of this kinase might be responsible for some "metformin-like" effect of polyphenols [351]. The effects on AMPK-phosphorylation were not further pursued. Metformin is a known AMPK-activator [19, 359]. Previous studies reported a capability of polyphenols to increase the AMPK-phosphorylation as well [71, 360].

2.8 Influence of glucose on the effects of polyphenols on pAkt *in vitro*

2.8.1 Background: Influence of glucose concentrations on pAkt *in vitro*

Hyperglycaemia the principal symptom of type 2 diabetes mellitus (T2DM) leads to vascular complications such as coronary artery disease, nephropathy and retinopathy [361]. Micro- and macroangiopathies are due to endothelial dysfunction (ED) [362].

In diabetes mellitus or metabolic syndrome, hyperglycemia can cause ED in a both direct and indirect manner. The direct effects of elevated glucose are increased osmotic stress, hyperglycemia-induced activation of the DAG/PKC (diacylglycerol/protein kinase C) signaling pathway (which in turn can lead to increased oxidative stress), N-acetylglucosamine-dependent modification of proteins, and non-enzymatic glycosylation. The indirect pathway involves an impaired balance in the production of growth factors, cytokines and vasoactive agents (e.g., TNF-α, IGF-1, EGF and VEGF) [363].

Numerous studies have shown that increased glucose concentrations have a significant impact on the vitality and growth of endothelial cells *in vitro*. Lorenzi et al. reported that persistent hyperglycemic conditions (20 – 40 mM glucose) may lead to delayed growth and disruption of the primary endothelial cells's cell cycle (HUVEC) [364]. In addition, it has been described that increased glucose concentration can induce apoptosis [365]. The negative role of advanced glycation end products in endothelial cells formed under hyperglycaemic conditions (30 mM glucose) had also been investigated. Thus, increased glucose levels can affect many cellular signaling pathways and pathophysiological mechanisms [366].

2.8.2 Results: Influence of glucose concentrations on pAkt *in vitro*

The *in vitro* model (C-2.4.1.4) mimicking diabetes relevant elevated glucose concentrations demonstrated significant alternation of Akt signaling. The responses of starved EA.hy926 cells to 1 µM insulin/ 10 µM histamine mixture following cultivation

under normoglycemic (LG, low glucose - 5.5 mM glucose) and hyperglycemic (HG, high glucose - 25.0 mM glucose) conditions differed from each other. The protein phosphorylation analysis through Western blot (pAkt Ser473) uncovered that Akt response was diminished under hyperglycemic conditions in comparison to the normoglycemic ones (Figure 32, n= 3).

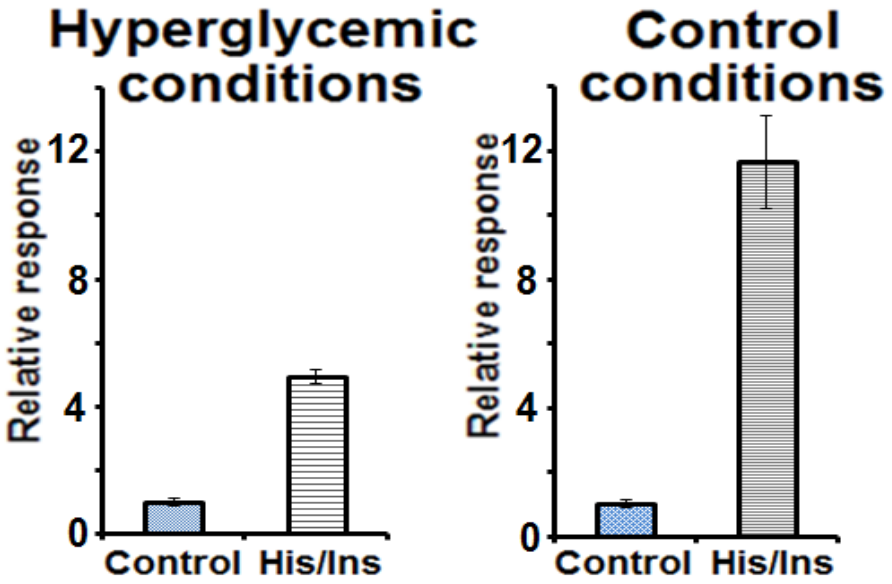


Figure 32: ***In vitro*** **model mimicking hyperglycemia: densitometric analysis of Akt phosphorylation on the Ser473 position. The columns indicate the mean values and mean deviations of the mean. Under hyperglycemic conditions (25 mM) the stimulation of Ea.hy926 cells with insulin/histamine mixture (His/Ins) caused 4.9 times increase in the phosphorylation status of Akt. In comparison, this response under control conditions (5.5 mM) was higher – around 12 times. Both responses were normalized to the negative controls – untreated cells (n= 3).**

Under hyperglycemic conditions Akt phosphorylation was increased 4.9 times in a response to the stimulation. The respective response under normoglycemic control conditions was 11.5 times. These responses were normalized to the negative control (untreated cells). The results were in accordance with the literature [248] and confirmed the impact of the glucose concentrations on Akt signaling in the employed *in vitro* model.

The short-term effects of polyphenols after 5 min incubation were analyzed by ELISA both at physiological conditions (LG), and in pathological hyperglycemic conditions (HG) *in vitro* in EA.hy926 cells. The influence on Akt-phosphorylation was evaluated in comparison with the corresponding control (HG or LG). The effects of resveratrol differed remarkably under both conditions (mean inhibition ± standard deviation). Resveratrol statistically significantly reduced the phosphorylation of Akt with 19.96 ± 8.83 % under HG ($p= 0.003$), while in contrast it caused only a minor reduction of pAkt under LG

conditions: 2.30 ± 10.19 % (n= 5, p= 0.9). Quercetin reduced statistically significantly Akt-phosphorylation with 36.46 ± 9.84 % for HG (p= 0.001) and with 26.23 ± 11.18 % for LG (p= 0.001). The inhibitory effect was clearly stronger under hyperglycemic conditions. The basal phosphorylation of the control in LG was slightly lower than in HG, but this difference (10.88 ± 11.58 %) was not statistically significant (p= 0.095 ANOVA, Figure 33).

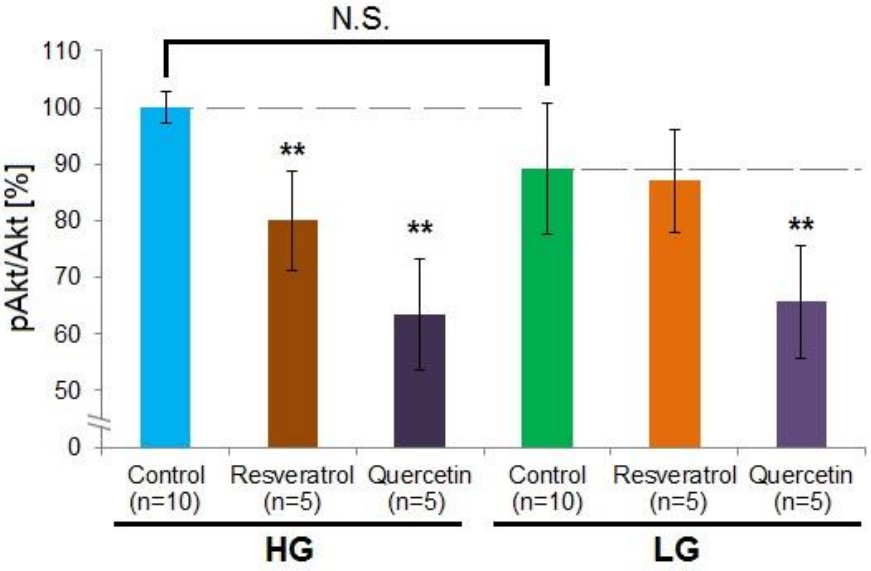


Figure 33: Influence of different glucose concentrations on the effects of the model compounds quercetin and resveratrol (10 µM) on the phosphorylation of Akt at Ser473 site in EA.hy926 cells. Columns represent mean values and standard deviations. Inhibitory effects of both polyphenols were remarkably different depending on the glucose concentrations. Resveratrol inhibited statistically significantly Akt-phosphorylation under hyperglycemic conditions (HG) (p= 0.003), while it showed practically no inhibitory effect under normoglycemic conditions (LG) compared to controls. Quercetin inhibited pAkt statistically significantly under both conditions (** p= 0.001), whereby its inhibitory activity was with around 10 % stronger under hyperglycemic conditions compared to normoglycemic ones. The phosphorylation of the control was slightly lower in LG with 10.88 ± 11.58 % compared to the HG control, but this difference was not statistically significant (N.S., p> 0.05, one-way ANOVA/Tukey HSD test).**

These results were obtained in the presence of growth factors in the cell culture medium. The effect of quercetin on Akt-phosphorylation was also studied in the absence of growth factors by incubating the cells in serum-free medium. In contrast to HG conditions, quercetin did not show any inhibition of the Akt-phosphorylation under LG (data not shown).

Subsequently, the effects of quercetin were tested on HUVEC cells in order to check if these findings are translatable for primary endothelial cells, which are considered a more realistic model reflecting the physiological state of organisms better compared to the immortalized cell lines (C-2.1.1).

Similar to the results obtained with EA.hy926, quercetin caused statistically significant reduction of Akt-phosphorylation under both HG (p= 0.001) and LG (p= 0.001) conditions in the presence of growth factors (one-way ANOVA/Tukey HSD test). No statistically significant difference between the controls of both conditions was detected, although Akt-phosphorylation seemed to be higher under LG than HG (Figure 34).

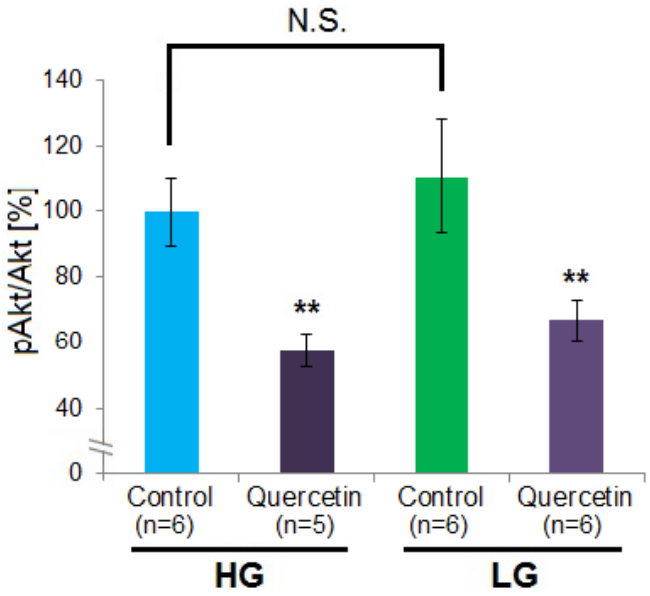


Figure 34: **Effects of quercetin on Akt-phosphorylation in HUVEC cells under both hyperglycemic-like (HG) and normoglycemic-like (LG) conditions. Columns represent mean values and standard deviations. The polyphenolic compound reduced the phosphorylation of Akt with 42.47 ± 5.08 % (HG), respectively 43.84 ± 6.11 % (LG) compared to controls. These effects were statistically significant (** p= 0.001, one-way ANOVA/Tukey HSD test). The difference between HG- and LG- controls was not statistically significant (N.S.).**

2.8.3 Discussion: Influence of glucose concentrations on pAkt *in vitro*

The comparison of the *in vitro* effects of quercetin and resveratrol on Akt-phosphorylation under hyperglycemic-like (HG) and euglycemic-like conditions (LG) suggested a possible influence of the elevated glucose concentrations on the effects of polyphenols on pAkt.

The basal phosphorylation status of Akt (corresponding to untreated cells) was higher under HG-conditions compared to LG in Ea.hy926 immortalized cells. In contrast, in primary HUVEC cells the basal Akt-phosphorylation under LG conditions tended to be

higher than this under HG. Both differences were not statistically significant. It has been reported that elevated glucose can either increase or decrease pAkt depending on the cell type. For instance, a study employing HUVEC cells showed that the activity of PI3K/Akt-pathway was reduced by exposure to HG (20 – 40 mM glucose) compared to LG (5 mM), which seemed to be in accordance with the present results obtained within the same type [367]. However, another investigation revealed that Akt-phosphorylation was higher under HG conditions (40.7 mM) in comparison to LG (5.7 mM) in retinal endothelial cells, due to increased Src-signaling (a non-receptor tyrosine kinase) [368]. Thus, different endothelial cells might react differently to elevated glucose concentrations which might contribute to different effects of polyphenols tested under HG and LG. Further reasons for the divergent results could be different concentrations and composition of growth factors (see C-2.1.2.1) in the media, passage dependence, and duration of the HG-exposure. Possible diversity of cellular signaling between the primary and immortalized cells cannot be ruled out as well.

Differential effects of the model polyphenols quercetin and resveratrol on pAkt observed in Ea.hy 926 cells still can be considered intriguing, although not confirmed for the tested compound quercetin in primary HUVEC cells. A previous study likewise showed that resveratrol exhibited more pronounced effects on Akt under HG than under LG [257]. In contrast to the present study authors investigated long term effects (12 h) and reported an activation of pAkt. In the present study short term effects were of interest. According to own results, long exposure of cells to resveratrol tended to increase Akt-phosphorylation as shown previously (see pilot study; B-2.1.2). The authors of that work [257] confirmed experimentally the involvement of PTEN (phosphatase and tensin homolog) and discussed a possible role of AMPK in the observed effects which may be applied to the present results as well. To our best knowledge the present study appeared to be the first one comparing the effects of quercetin on the phosphorylation status of Akt in endothelial cells under different glucose levels.

2.8.4 Conclusions: Influence of glucose concentrations on pAkt *in vitro*

The data from the present study revealed differential effects of polyphenols on the Akt-phosphorylation under different glucose concentrations *in vitro* and may broaden the understanding for the importance of these nutrients and their mechanisms of action in T2DM and support their role in alleviating HG-induced endothelial dysfunction and consequent diabetic complications. Further research has to be conducted to verify the physiological relevance and to elucidate the underlying mechanisms responsible for these results.

The outcome from the presented investigation might give some insights about the rationale for using polyphenols as nutraceuticals in management of T2DM and its co-morbidities [369].

3 Effects of polyphenols on DPP IV activity and expression

3.1 Effects of polyphenols on the enzymatic activity of DPP IV

3.1.1 Background: Polyphenols and DPP IV activity

As already mentioned (B-1.2.1), DPP IV is an enzyme responsible for the inactivation of incretin hormones (as GLP-1) and therefore has an important clinical significance as a target for treatment of T2DM [187]. In addition to the approved DPP IV inhibitors (i.e. sitagliptin) it was described that some natural compounds are capable to reduce the activity of DPP IV [370]. An extensive literature research indicated that various polyphenols are reported as potent DPP IV inhibitors [215, 370, 371].

The *in vitro* studies published so far show that some polyphenols act as DPP IV inhibitors in the micromolar and nanomolar concentration range. This was discussed in detail under B-1.3.3.

Considering results from the literature review and the known outcome from the clinical study for the action of PYC (B-1.3.2) on DPP IV, the effects of individual monomeric ingredients of the extract and their metabolites on the enzymatic activity of purified DPP IV was investigated. The inhibitory potential of various polyphenols was additionally examined in an *in vitro* model.

3.1.2 Results: Polyphenols and DPP IV activity *in vitro*

Initially, a comprehensive screening regarding the *in vitro* inhibitory potential on the activity of DPP IV of polyphenols with diverse structure was performed. Thirty representatives from the following subclasses were included in the study: flavanones, flavones, flavonols, flavanonols, flavan-3-ols (catechins), isoflavones, stilbenoids and phenolic acids. In addition, five microbiota-generated metabolites of polyphenols were investigated.

The results indicated no or only a minor reduction in the activity of DPP IV by the investigated polyphenols at a concentration of 5 µM compared to the initial 100 % enzyme activity in the approach utilizing purified human recombinant DPP IV (Figure 35).

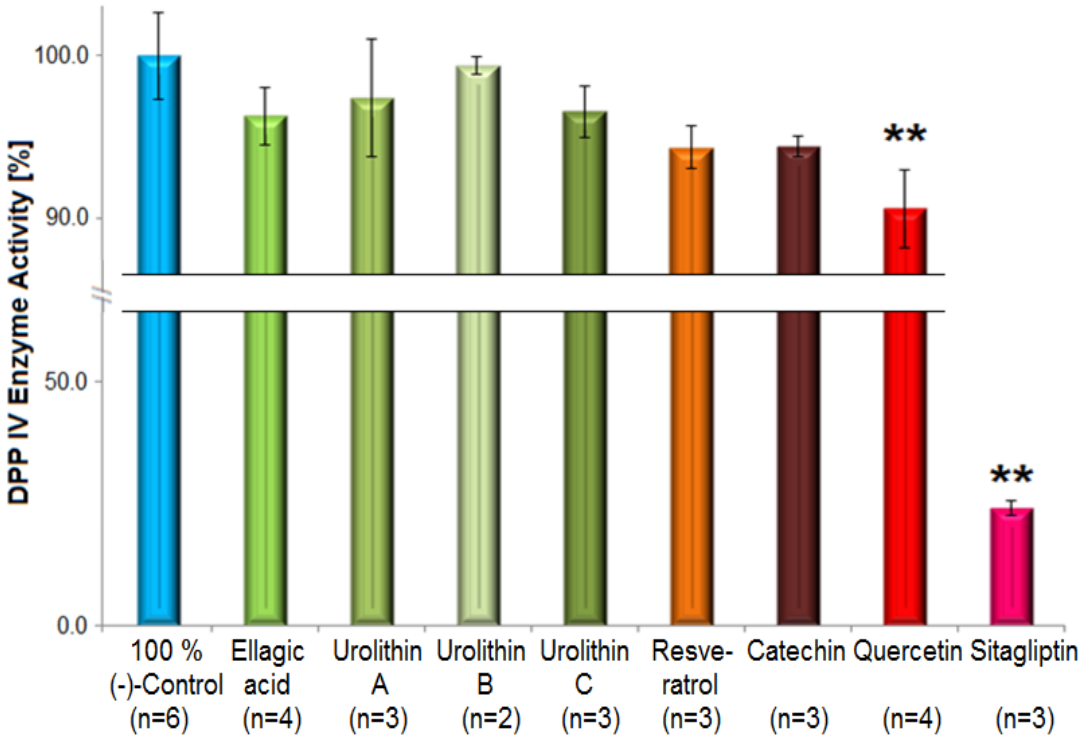


Figure 35: Effects of polyphenolic compounds tested at a concentration of 5 µM on the initial enzymatic activity in the purified human recombinant DPP IV approach. The columns indicate the mean values and mean deviations of the mean (in %). Quercetin, resveratrol, and (+)-catechin were shown to possess the clearest inhibitory potential among all tested compounds. The effects of these substances were small and only this of quercetin was statistically significant (* p= 0.002, one-way ANOVA with Tukey's post-hoc test). In contrast to the investigated substances, the reference inhibitor sitagliptin (50 nM) caused robust enzyme inhibition of 75.37 ± 1.42 % (n= 3).

Inhibitions (mean ± mean deviation of the mean) were small and the most pronounced were caused by quercetin (9.42 ± 2.40 %), resveratrol (5.65 ± 1.28 %), and (+)-catechin (5.63 ± 0.62 %). Among them only the effect of quercetin was statistically significant (p= 0.002, one way ANOVA with Tukey's post-hoc test). The metabolites of polyphenols which were created by bio-transformation through gut microbiota urolithin A, B, C and D, as well as delta-(3,4-dihydroxy-phenyl)-gamma-valerolactone (M1) exhibited very low or no inhibitory potential. Taxifolin and caffeic acid which along with (+)-catechin are monomeric ingredients of PYC also did not show any remarkable inhibitory effects on DPP IV. The obtained results for all tested compounds can be found in the Supplement Section D-4.1. In comparison to the tested compounds the reference substance (50 nM Sitagliptin) caused a strong decrease in the DPP IV activity of 75.37 ± 1.42 % (n= 3).

In order to estimate better the clinical relevance for the effects of the most potent among the investigated compounds quercetin and resveratrol, both of them were investigated in

the modified approach of DPP IV inhibition assay utilizing human serum probes as a source of the enzyme and the assay was validated with the reference substance sitagliptin (C-3.1.3.2).

After consideration of the initial fluorescence of the serum spiked with quercetin and resveratrol, the results indicated that both compounds have minor effects on the DPP IV activity, respectively mean decrease and mean deviation of 4.21 ± 2.32 % and 2.93 ± 0.86 % (n= 2).

3.1.3 Discussion: Polyphenols and DPP IV activity *in vitro*

The experiments performed with human recombinant DPP IV showed that the investigated compounds at a concentration of 5 µM had only weak effects on DPP IV activity. Quercetin, resveratrol and (+)-catechin were the substances with the clearest effects, but among them only the inhibition by quercetin was statistically significant. However, quercetin and resveratrol exhibited only a minor influence on the activity of the enzyme in the serum sample approach.

The obtained results are in contradiction with the data published so far. Previous studies claim that many monomeric polyphenols are capable to inhibit DPP IV potently (B-3.1.1, B-1.3.3). It was reported the IC_{50} values of the most potent polyphenolic compounds are in the low micromolar or nanomolar concentration range. To compare, in the present study substances at a 5 µM concentration were tested. Although this concentration was much higher (> 8000 to 2 fold) than the described ones for a 50 % inhibition of the enzyme activity (IC_{50}) in the published investigations [215, 221], it was not possible to observe an inhibition stronger than 10 %. A conceivable reason for the difference between the current and the published results could be the use of different type of enzyme (i.e. porcine DPP IV [215, 221]) instead of human recombinant one used in the present study as despite the high homology some functional differences between both were observed [372]. In addition, the determined IC_{50} might be strongly dependent on the enzyme and substrate concentrations [373]. Indeed, in the study of Fan et al. [215] a porcine DPP IV enzyme at a final concentration of 1 ng/mL was used. This value is clearly lower than the mean physiological concentration (753 ng/mL) and the individual serum concentrations of DPP IV, which were detected in the performed clinical study (B-1.3.2.1). Likewise, other studies reported DPP IV concentrations in the similar range, which had already been discussed (B-1.3.3). Another possible reason for the contradictory results could be a neglect of potential interactions of polyphenols with the experimental setup, for instance coupled luciferase [374] (luminescent assays) or fluorescent signal (fluorescence-based assays). Evidence is available in the literature that polyphenols might reduce the intrinsic fluorescence of some serum proteins [375]. That can influence the result of such *in vitro* studies and must be considered. In this regard, investigations for the impact of polyphenols

on the fluorescence readouts and presence of eventual methodological artifacts were carried out (B-4.2).

The contradiction between the results from previous studies and the present one along with the discussed reasons about that rise a question for the clinical significance of polyphenols as DPP IV inhibitors.

The results obtained from the *in vitro* screening does not support the results from previous studies, but confirms the outcome of the performed clinical study (B-1.3.2.2) that the investigated extract did not possess any DPP IV inhibitory effects as well as its ingredients and their metabolites indicated *in vitro*. Although the slight reduction caused by the dietary flavonoid quercetin was statistically significant this effect was way too small (< 10 %) compared to the one of the referent substance sitgliptin which indicated around 75 % reduction of the initial DPP IV activity at much lower nanomolar concentrations. Thus, it is not likely that quercetin possess any clinically relevant inhibitory potential.

As a conclusion, the results indicated that the investigated compounds are not likely to be direct inhibitors of DPP IV enzymatic activity neither *in vitro* nor in human subjects.

3.2 Effects of polyphenols on the expression of DPP IV/ CD26

3.2.1 Background: DPP IV/CD26 Expression

Dipeptidyl peptidase IV (DPP IV, also named CD26) is considered a pleiotropic molecule. Along with being protease it exhibits further enzymatic activities, paracrine and endocrine functions and thus might influence multiple cellular targets and pathophysiological processes [204].

Changes in serum levels or expression of DPP IV were investigated with reference to different morbidities, including cancer, inflammatory, autoimmune, and cardiovascular diseases. Accumulating knowledge from recent years indicated a straightforward relationship between the development of T2DM and its associated metabolic syndrome and dysregulation in DPP IV serum levels [376]. In particular it was demonstrated that serum DPP IV concentrations were elevated in insulin resistant and obese patients in comparison to healthy individuals [206, 377]. A positive correlation between DPP IV expression levels in adipocytes and body mass index (BMI) of participants was established. In addition, a recent studies showed the crucial role of DPP IV in human adipocytes' metabolism and identified adipose tissue as a relevant source of circulating DPP IV in serum [206, 377, 378]. Also, it had been suggested that glucose concentrations might influence *in vitro* production of DPP IV by adipocytes or CaCo-2 cells [379, 380]. These observations were

relevant for the present study as all experiments were performed under high glucose conditions (25 mM glucose).

In addition to adipocytes, T-cells were described to express DPP IV and were characterized to be responsible for elevated serum concentrations in diabetic patients. Furthermore, DPP IV levels were found to be associated with the status of glycemic control of T2DM individuals [381].

Studies investigating DPP IV expression also considered diabetes-related pathological states. For instance, myocardial infarction may occur as a severe consequence of T2DM macroangiopathy. A translational study showed that down-regulated expression of DPP IV can ameliorate cardiac function after myocardial infarction [382]. In a diabetes relevant cell-based model it was demonstrated that hyperglycemia can induce expression of DPP IV in glomerular endothelial cells [383].

As several studies indicated that DPP IV serum levels and expression are increased in T2DM and its co-morbidities it was conceivable that down-regulation of DPP IV-expression and/or reduction of its release might be beneficial and a successful therapeutic strategy.

The regulation of DPP IV on pre-transcriptional and transcriptional level was not fully clarified. It was suggested to be complex and probably cell type specific [376]. It was shown that the DPP IV promoter can be influenced by various DNA-binding elements and transcription factors and the regulation mechanism may vary among different tissues [384]. Multiple transcription factors were shown to be involved in the process. Hepatocyte nuclear factor-1α (HNF-1α) was described to mediate at least at part the effect of glucose on the DPP IV gene expression *in vitro* [380]. Mutants of HNF-1α and HNF-1β that occur in maturity onset diabetes of the young (MODY) can down-regulate DPP IV levels in a cell-based model [385]. Additional transcription factors participating in the regulation of DPP IV are NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), SP-1 (specificity protein 1), STAT (signal transducer and activator of transcription), AP-1 (activator protein-1) factor [386]. As hypoxia altered the expression of DPP IV in pre-adipocytes an important role of hypoxia-inducible factor (HIF-1α) was also suggested [387].

Due to the involvement of DPP IV in several cellular process and pathologies the modulation of its expression by pharmacological agents is of high interest. Discovery of novel agents capable to influence DPP IV expression might uncover new physiological mechanisms, signaling pathways, and target molecules involved in the gene transcription and thus to expand the basic knowledge. And *vice versa*: defined targets taking part in the regulation of DPP IV expression can be advantageous for development of lead structures of active compounds. In this regard, it was shown that interferons (α, β, γ) and all-trans-retinoic acid (ATRA) can up-regulate DPP IV levels in B-cells. The molecular mechanism

proposed by the authors included STAT-1-alpha activation followed by nuclear translocation and binding to the GAS (gamma interferon-activated site)-response element of the DPP IV promoter [388].

DPP IV exists not only as a membrane-bound, but also as a soluble (serum) form [204]. The latter can be released from the cell membrane by shedding. TNF-α and insulin were described as agents promoting liberation of DPP IV from adipocytes [206, 379].

The surface expression of DPP IV might also be important for its serum levels. In this regard adenosine was described to reduce the DPP IV surface expression in HT-29 colon cancer cells [389, 390]. Forskolin influenced cellular trafficking and caused internalization of already membrane expressed DPP IV [391]. This process was shown to be cAMP-independent although forskolin is well-known activator of adenylate cyclase [392]. In addition, the cytokine TGF-β1 downregulated the CD26/DPP IV surface expression in T-cells [393].

In summary the described processes of gene expression, intracellular transport, cell surface expression and release of DPP IV appear to be important for its serum levels and enzymatic activities.

Since dietary polyphenols may contribute to the prevention and treatment of T2DM, the potential of polyphenols to influence the release/secretion and expression of DPP IV is of high interest. It was suggested that polyphenols might modulate the expression of DPP IV by interaction with the transcription factor HNF-1α [394].

Since the clinical study described in the present work (B-1.3.2) revealed the effects of French maritime pine bark extract (PYC) on the serum levels of DPP IV in humans this effect was investigated in more detail.

3.2.2 Results: Polyphenols and DPP IV Expression

Effects of three groups of compounds on the total protein expression of DPP IV were analyzed in an *in vitro* model of 3T3-L1 adipocytes (C-3.2.3). The mean level of DPP IV in differentiated, but untreated cells was 17.05 ± 3.35 ng DPP IV/mg protein (n= 24). In comparison, DPP IV expression in undifferentiated 3T3-L1 fibroblasts was around 2 ng DPP IV/mg protein. All compounds were tested at a concentration of 10 µM for 72 hours, the cell culture medium containing the respective substance or vehicle was changed every 24 hours.

The first group included ingredients and metabolites of the French maritime pine bark extract (PYC). There was no statistically significant influence on the total protein expression of DPP IV in murine adipocytes neither by PYC-constituents nor by their microbiotia-generated metabolites (n= 6 – 15; Table 9A).

Table 9.: Descriptive statistics for the effects of different natural compounds on the DPP IV protein expression: A. Ingredients and metabolites of Pycnogenol®. B. Dietary polyphenols. C. Ellagic acid and urolithins. D. Other natural compounds. Abbreviations: S.D. – standard deviation, PMA – Phorbol ester, ATRA – All-trans-retinoic acid.

Ingredients and metabolites of Pycnogenol®					
A	Mean [%]	S.D. [%]	Median [%]	n	Statistical significance
Control	100.00	2.62	100.00	29	-
Taxifolin	111.06	10.49	113.46	12	n.s.
(+)-Catechin	103.16	7.85	104.42	8	n.s.
M1	95.02	8.14	96.10	15	n.s.
M2	100.90	17.72	102.14	6	n.s.
Caffeic acid	100.94	7.12	99.59	8	n.s.
Ferulic acid	90.63	12.43	91.73	6	n.s.
Dietary polyphenols					
B	Mean [%]	S.D. [%]	Median [%]	n	Statistical significance
Control	100.00	6.55	101.76	19	-
Apigenin	90.33	8.45	89.23	7	n.s.
Luteolin	91.94	13.74	90.25	8	n.s.
Quercetin	98.11	9.11	95.54	4	n.s.
Naringenin	101.99	3.77	101.78	3	n.s.
Genistein	108.38	17.51	108.62	4	n.s.
Resveratrol	100.67	2.07	100.07	4	n.s.
Ellagic acid and urolithins					
C	Mean [%]	S.D. [%]	Median [%]	n	Statistical significance
Control	100.00	6.16	102.27	18	-
Ellagic acid	96.47	0.32	96.47	2	n.s.
Urolithin A	101.64	8.73	101.08	8	n.s.
Urolithin B	92.20	2.74	92.20	2	n.s.
Urolithin C	95.40	16.35	88.09	6	n.s.
Urolithin D	88.16	11.31	92.54	5	n.s.
Other natural compounds					
D	Mean [%]	S.D. [%]	Median [%]	n	Statistical significance
PMA	79.54	8.57	82.25	7	$p < 0.01$
ATRA	115.57	18.06	112.79	7	$p < 0.01$

Only M1 and ferulic acid showed slight tendencies to reduce DPP IV expression by 4.98 ± 8.14 %, respectively 9.37 ± 12.43 % compared to the controls. Taxifolin tended to increase the protein expression of DPP IV. One-way ANOVA with Tukey HSD test revealed that these effects were not statistically significant.

Another group that underwent analysis for any influence on the expression of DPP IV was dietary polyphenols with different structures. Flavones luteolin and apigenin, flavonol quercetin, flavanone naringenin, isoflavone genistein and stilbenoid resveratrol were described to be often administrated with food [82, 395]. For this reason, their effects on the DPP IV expression were investigated, but they showed no prominent effects on DPP IV levels *in vitro* (Table 9B). Among these compounds only apigenin and luteolin tended to reduce the expression of DPP IV with respectively 9.67 ± 8.45 % and 8.06 ± 13.74 %, while in contrast genistein-treatment caused a slight increase of 8.38 ± 17.51 % compared to the control. None of the mentioned effects was statistically significant (n= 3 – 7).

Ellagic acid and its bacterial metabolites urolithins did not induce any statistically significant changes of the enzyme's expression *in vitro* (n= 2 – 8). Slight tendencies for a reduction of the DPP IV levels were shown for urolithin B (7.80 ± 2.74 %), C (4.60 ± 16.35 %), and D (11.84 ± 11.31 %). In contrast, ellagic acid and urolithin A did not cause any effect on the expression of DPP IV on the protein level (Table 9C).

Phorbol ester (PMA) at a 1 µM concentration caused a statistically significant down-regulation of the DPP IV protein expression by 20.46 ± 8.57 % (n= 7). As the effects of PMA were statistically significant and reproducible in independent experiments, it was considered as a positive control in the experimental set-up. Along with that the effects of all-trans retinoic acid (ATRA) (10 µM, 48 hours) were investigated as it had been described to increase the intracellular and surface expression of DPP IV in tumor B cells [388]. The results showed statistically significant up-regulation (mean increase of 15.57 ± 18.06 in regard to the control, n= 7) of the total DPP IV expression ($p < 0.01$, one-way ANOVA) (Table 9D).

The DPP IV release by 3T3-L1 adipocytes in conditioned medium was also investigated. After cell culture supernatants were concentrated and dialysed (C-3.2.3) the DPP IV concentration was measured. The concentration of released DPP IV was estimated to be around 4 pg/mL – a value below the limit of quantification of the ELISA. As this value was lower than expected the model was considered as inappropriate for further investigation of the impact of polyphenols on DPP IV liberation on a cellular level.

3.2.3 Discussion: Polyphenols and DPP IV Expression

Regarding the benefits of polyphenols in T2DM their potential influence on the expression of DPP IV is of considerable interest [371, 396-398]. The present work investigated the effects of 17 individual polyphenols and their metabolites on the total protein expression of

DPP IV in 3T3-L1 differentiated adipocytes. The compounds with slight, but non-statistically significant tendencies for reduction of DPP IV levels compared to controls were urolithins C and D, apigenin, luteolin, and ferulic acid. In addition to their minor activities urolithin D had never been detected in human plasma [399] and therefore these effects cannot be expected to be clinically relevant.

In general the present data is in discordance with the results from some already published articles claiming that polyphenols can down-regulate DPP IV expression [371, 396] and in accordance with other ones revealing respectively no activity of polyphenolic compounds [397, 398]. Any discrepancies might be due to e.g. differences in the experimental set-up and diversity in the regulation of DPP IV gene transcription among different tissues and cell types.

In the present work effects of individual compounds were investigated in contrast to previously published studies testing mainly complex polyphenolic extracts. Along with effects of either anthocyanins or proanthocyanidins on the protein expression of DPP IV the individual compound resveratrol was investigated in CaCo-2 cells [371]. In accordance with the present results resveratrol was inactive. Anthocyanin and proanthocyanidin fractions reduced the DPP IV protein levels statistically significantly [371, 396]. On the contrary, two studies reported no effects of a polyphenol extract of hibiscus on the DPP IV protein level in HK-2 cells [397, 398].

Proanthocyanidins were not included in the present screening as its object was an investigation of monomeric compounds. Furthermore the interpretation of *in vitro* results regarding extracts' effects can be challenging due to their complex and not always fully-defined content and low or missing serum concentrations of their ingredients upon intake.

The data from the present screening suggested that the investigated monomeric components of PYC did not cause any statistically significant changes on the DPP IV total protein expression in adipocytes. Therefore, the *in vivo* effects of PYC might be due to other so far unknown metabolites or constituents of the extract. A possible synergistic effect between the compounds on the expression of DPP IV cannot be excluded. Alternatively, the mechanism of PYC's action might not include an impact on the intracellular DPP IV levels, but rather influence on its surface expression and shedding.

A study showing that apigenin modulated the membrane expression of DPP IV in HT-29 cell based model suggested that the DPP IV surface expression might be a possible target of polyphenols [400].

Presently there is no information in the literature that polyphenols might affect the process of DPP IV release. However, the membrane proteins' shedding is mediated by the ADAM family of metalloproteinases [401]. In particular, the liberation of DPP IV was described to be mediated by matrix metalloproteinases (i.e. MMP I, MMP IX) [402]. PYC and its

metabolites were reported to reduce the expression and/or activity of some MMPs and ADAM members [180, 403]. In addition, a study showed that the release of DPP IV can be increased under oxidative stress conditions, although the mechanism of this process in not completely clarified [404]. As polyphenols are described as antioxidants [310] they might modulate the process of shedding.

The *in vitro* model utilizing 3T3-L1 adipocytes did not appear to be suitable for investigation of DPP IV shedding process in the present study. Concentrations of DPP IV released by 3T3-L1 adipocytes into cell culture medium were estimated around 4 pg/mL which is in conflict with the previously published data claiming concentrations of approximately 20 – 60 pg/mL in cell culture supernatant of the same cell type [379]. This might be explained with the low expression of DPP IV on the surface of the used cells. An appropriate *in vitro* model capable to reflect the process of shedding of DPP IV on cellular level is demanded. Apart from adipocytes described as a major source of soluble DPP IV it has been shown that T-lymphocytes can release DPP IV and can significantly contribute to the serum circulating DPP IV exists [381, 405-407]. Recently the role of T-cells as a source of soluble DPP IV was investigated in T2DM related studies [381, 407].

The published information about modulators of DPP IV expression is sparse, but ATRA (all-trans-retinoic acid) was shown to increase the protein expression of DPP IV in tumor B cells [388]. In the present study employing adipocytes DPP IV protein levels were statistically significant increased by ATRA in comparison to controls. This result is in accordance with the result described in the cited study.

PMA (phorbol ester) induced statistically significant downregulation of the total protein expression of DPP IV in adipocytes around 20 %. To the best of our knowledge such an effect for this compound has not been described before. A previous study showed that phorbol ester was inefficient in influencing the gene transcription and DPP IV mRNA (messenger RNA) level in human glomerular epithelial cells [408]. The outcome from the present work might stimulate further research about the signaling pathways involved in the regulation of DPP IV gene transcription.

3.2.4 Conclusion: Polyphenols and DPP IV Expression

The present results indicated that the monomeric components of PYC and its metabolites are not likely to contribute to the reduction of DPP IV serum concentrations *in vivo* through down-regulation of total protein expression of DPP IV in adipocytes. Further research is needed to establish if polyphenolic compounds can influence the surface expression and release of DPP IV.

4 Fluorescence interactions of polyphenols in different environments

4.1 Background: Fluorescence interactions

Polyphenols are object of comprehensive research programs. Such an investigation is quite challenging because of the chemical instability of these compounds [272] and their potential to interact with the assay set-ups which might result in misleading conclusions [409, 410].

In order to verify the DPP IV inhibitory potential of polyphenols as described in the literature and to estimate the activity of further compounds of interest, *in vitro* investigations involving 35 compounds were conducted (B-3.1). During the screening some unusual results were observed. For instance, the flavones 6-methoxyflavone, 6-hydroxyflavone, 7-methoxyflavone, 3-hydroxyflavone, 3-hydroxy-4'-methoxyflavone, and genistein indicated increased fluorescence values (Supplement Section D-4.1). Anyway, an *in vitro* activation of the enzyme through those compounds was unlikely and not described in the literature so far. In addition, quercetin seemed to decrease the fluorescence in human serum at the starting point of the measurement, even before addition of the DPP IV substrate (H-Gly-Pro-AMC). Therefore, it was assumed that some polyphenols might interact with the fluorescent signal and thus to alter the results of the assay. Respective fluorescence measurements were performed in order to confirm this suspicion. Quercetin, 6-methoxyflavone, urolithin A and B seemed to interfere with the fluorescent readouts in the strongest way among the investigated 35 compounds and therefore were chosen for further analysis in order to quantify these effects under defined conditions and to determine if they are reproducible and statistically significant.

Fluorescence measurements were conducted in experimental environments which were possibly closest to those of the classic and modified DPP IV inhibitor screening assay (C-3.1), listed under C-4.3. Thus, it was possible to compare the effects of polyphenols and to conclude if they are universal or specific attribute due to any of the matrices. Additionally, investigations were performed both in the presence or absence of the fluorescent product AMC in order to elucidate, whether effects of polypehnols were dependent or independent from it. Last but not least the fluorescence was measured before (0 min) and after (30 min) incubation at 37 °C, corresponding to the starting and ending points of the assay and revealed whether the temperature influenced readouts and whether it changed interactions, i.e. by influencing the compounds' stability.

4.2 Results: Fluorescence interactions

The measurements performed before (0 min) and after (30 min) incubation at 37 °C were overall similar. Shown are results after 30 min incubation corresponding to the endpoint of the assay. Results for the initial time point are represented in the Supplement, Section D-5. Effects on fluorescence were investigated in assay buffer, DPP IV solution, and human serum. Different conditions were signed as following: **no AMC, 0 min** – the absence of AMC, before incubation, **AMC, 0 min** – the presence of AMC, before incubation, **no AMC, 30 min** and **AMC, 30 min** – the absence and the presence of AMC, respectively, both after incubation.

Quercetin

Fluorescence effects in assay buffer

In the presence of AMC, quercetin at a 5 µM concentration quenched the AMC-fluorescence and slightly reduced its value in relation to the blank by 3.92 ± 2.85 % and this change was statistically significant ($p < 0.05$) (Figure 36, Quercetin, Buffer). In the absence of AMC, quercetin did not have any effect on the fluorescence (Figure 37, Quercetin, Buffer).

Fluorescent effects in DPP IV solution

Quercetin (5 µM) slightly reduced the fluorescent signal by 2.78 ± 2.31 % at 30 min, AMC and by 1.88 ± 1.55 % at 30 min, no AMC. Although this reduction was not statistically significant, it persisted under all investigated conditions (Figure 36, Quercetin, DPP IV; Figure 37, Quercetin, DPP IV, and Supplement, Section D-5, Table 1.2).

Fluorescent effects in human serum

At 30 min, AMC quercetin decreased fluorescence by 6.75 ± 2.78 % ($p < 0.01$) (Figure 36, Quercetin, Serum), and at 30 min, no AMC by 5.72 ± 1.99 % ($p < 0.01$) (Figure 37, Quercetin, Serum). As obvious, in presence of AMC this quenching effect was slightly bigger compared to the one established in the absence of AMC. It also seemed that incubation at 37 °C for 30 minutes decreased the quenching effect of quercetin to only a small extent (Supplement, Section D-5, Table 1.3).

Urolithin B

Fluorescent effects in assay buffer

Urolithin B did not cause any changes under any of the tested conditions in assay buffer (Figure 36, Urolithin B, Buffer; Figure 37, Urolithin B, Buffer).

Fluorescent effects in DPP IV solution

Urolithin B in the tested concentration did not cause any significant changes in fluorescence in the presence of purified DPP IV overall (Figure 36, Urolithin B, DPP IV; Figure 37, Urolithin B, DPP IV).

Fluorescent effects in human serum

Urolithin B strongly interfered with the fluorescence in human serum. Under all tested conditions 5 µM urolithin B exhibited statistically significant increase ($p < 0.01$) of the fluorescence in comparison to the experimental blank: 9.83 ± 2.10 % at 30 min, AMC (Figure 36, Urolithin B, Serum); 12.98 ± 2.97 % at 30 min, no AMC (Figure 37, Urolithin B, Serum). It seemed that before the exposure to temperature these effects were to some extent stronger (Supplement, Section D-5, Table 1.3).

Urolithin A

Fluorescent effects in assay buffer

In the presence of AMC there were no differences between urolithin A and the blank's fluorescence (Supplement, Section D-5, Table 1.1). Without AMC urolithin A slightly increased the fluorescence compared to the control with 4.35 ± 1.29 %. Nonetheless this change was not statistically significant.

Fluorescent effects in DPP IV solution

As the structurally related urolithin B, urolithin A did not cause any significant changes in fluorescence in the presence of purified DPP IV overall (Supplement, Section D-5, Table 1.2).

Fluorescent effects in human serum

Urolithin A also showed interference with the fluorescent signal in serum, although its effects were not as strong and as those of urolithin B (Supplement, Section D-5, Table 1.3). In the presence of AMC (30 min, AMC) urolithin A tended to increase the fluorescence compared to the blank by 2.67 ± 1.29 %, although not statistically significantly. In the absence of AMC (30 min, no AMC), it was a statistically significant increase of the fluorescence by Urolithinn A by 4.43 ± 2.81 % ($p < 0.05$).

6-Methohyflavone

Fluorescent effects in assay buffer

In buffer, under all tested conditions in the presence or in the absence of AMC, the fluorescence of 6-MF (6-methoxyflavone) at a concentration of 5 µM was statistically significantly higher than the blank ($p < 0.01$). The fluorescence was increased by 6-MF as following: by 5.44 ± 2.17 % at 30 min, AMC (Figure 36, 6-MF, Buffer); by 10.94 ±

3.28 % at 30 min, no AMC (Figure 37, 6-MF, Buffer). As obvious, the fluorescence increase caused by 6-MF was higher in the absence of AMC, than in the presence of it.

Fluorescent effects in DPP IV solution

6-MF increased the fluorescence under all tested conditions compared to the blank and these changes were statistically significant ($p < 0.01$). The increases caused by 6-MF at a concentration of 5 µM were 7.43 ± 4.22 % at 30 min, AMC; 10.45 ± 4.37 % at 30 min, no AMC. Similarly to the approach with the assay buffer, the effect of 6-MF was more distinct in absence of AMC (Figure 36, 6-MF, DPP IV), than in presence of it (Figure 37, 6-MF, DPP IV).

Fluorescent effects in human serum

In serum, 6-MF had no statistically significant influence on the fluorescence overall (Figure 36, 6-MF, Serum; Figure 37, 6-MF, Serum).

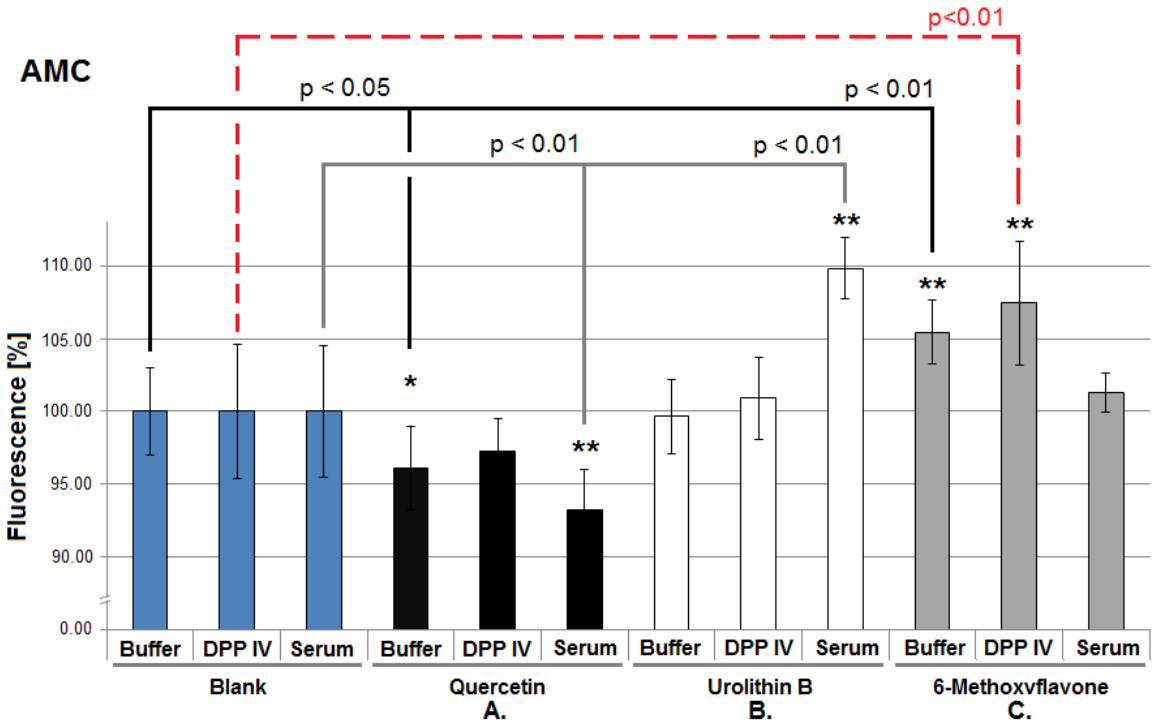


Figure 36: **Comparison of the influence of quercetin, urolithin B and 6-methoxyflavone at a concentration of 5 µM on the fluorescence ($\lambda_{excitation/emission}$ = 360 nm/460 nm) in different environments (buffer, DPP IV, and serum) in the presence of AMC. Columns represent means and standard deviations obtained from eight experiments (* p< 0.05, ** p< 0.01, n= 8). Quercetin quenched the fluorescence in all three cases. This effect was strongest in human serum – statistically significant decrease by 6.75 ± 2.78 % (p< 0.01) regarding the control. Urolithin B statistically significantly increased the fluorescence in serum by 9.83 ± 2.10 % (p< 0.01). 6-Methoxyflavone caused statistically significant rises (p< 0.01) in the fluorescence both in assay buffer and in the presence of DPP IV (respectively with 5.44 ± 2.17 % and 7.43 ± 4.22 % regarding the control).**

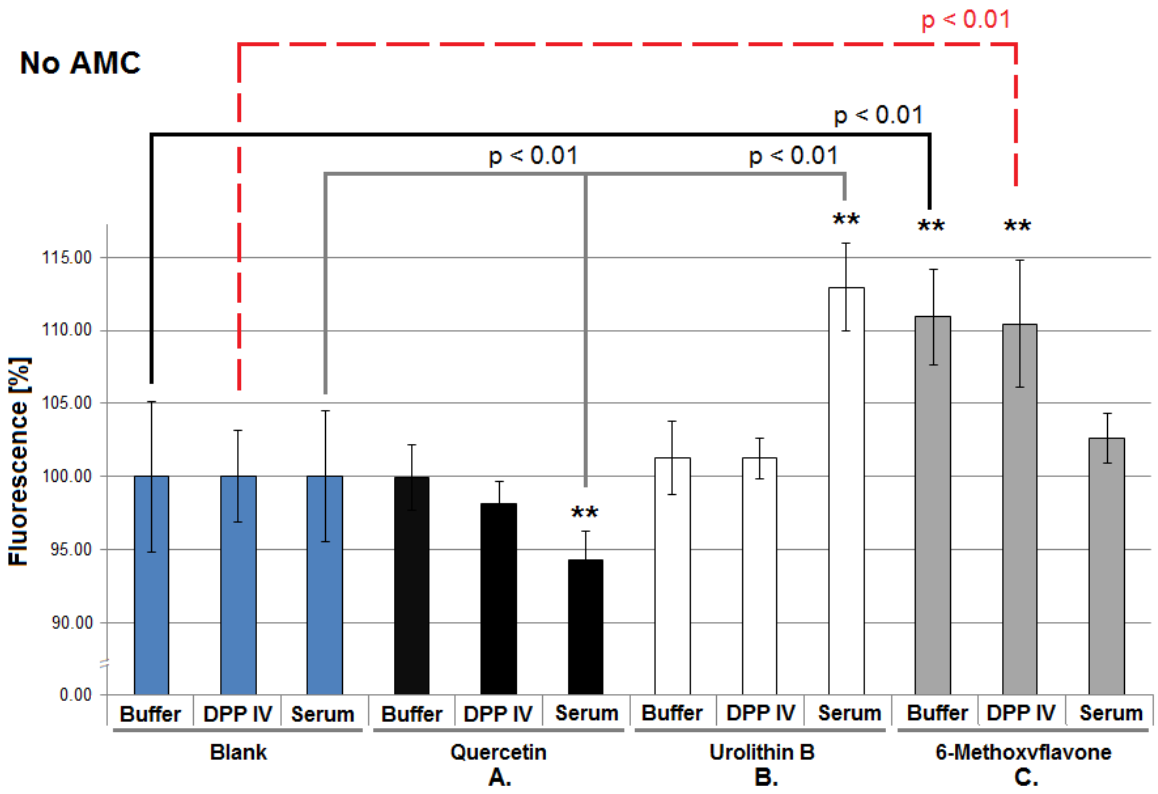


Figure 37: **Comparison of the fluorescent effects ($\lambda_{excitation/emission}$ = 360 nm/460 nm) of quercetin, urolithin B and 6–methoxyflavone at a concentration of 5 µM in different environments (buffer, DPP IV, and serum) in the absence of AMC. Columns represent means and standard deviations obtained from eight experiments (* p< 0.05, ** p< 0.01, n= 8). Quercetin quenched the fluorescence statistically significantly (p< 0.01) in serum by 5.72 ± 1.99 % regarding the control. Urolithin B increased the fluorescence in serum statistically significantly by 12.98 ± 2.97 % (p< 0.01). 6–Methoxyflavone raised the fluorescence statistically significantly (p< 0.01) both in assay buffer and in DPP IV solution (respectively with 10.94 ± 3.28 % and 10.45 ± 4.37 % regarding the controls).**

4.3 Discussion: Fluorescence interactions

The obtained results confirmed the suspicion that some polyphenolic compounds might interact with the fluorescence-based assay for DPP IV inhibition and in this way to change its outcome. The data indicated that 6-MF, Urolithins A and B were capable to increase the fluorescence readouts, while quercetin tended reduce them. During the study the role of different conditions for the described interactions was investigated: the experimental environment, the presence of the fluorescent product AMC, and the temperature.

Among all investigated matrices most significant interactions were detected within human serum. For instance, under all tested conditions in serum quercetin caused quenching and reduced the fluorescence statistically significantly ($p < 0.01$) compared to the blank. For quercetin such effects were detected in all tree matrices although they were not statistically significant in DPP IV solution. For the rest of the compounds outcomes in serum were different than those in assay buffer or DPP IV. Both urolithin A and B increased the fluorescence in serum notably, which was not the case in the other environments tested. On the contrary, 6-MF exhibited an intrinsic fluorescence in assay buffer and DPP IV, but had no prominent effects in serum. These differences might be explained with the complex composition of serum in comparison to the other matrices.

The anticoagulant warfarin was reported to exhibit similar fluorescent behavior as one observed for urolithins in the present study [411]. Namely, the fluorescence of this drug was notably enhanced due to its binding to human serum albumin (HSA). Considering that certain structural homologies between warfarin and urolithins exist, similar interaction might at least partly explain the observed increase of fluorescence in human serum.

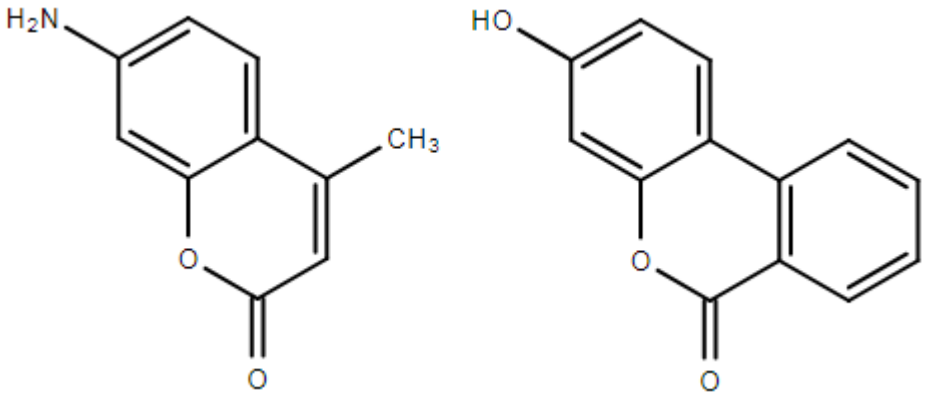
The fluorescent product AMC had considerable influence on the fluorescent effects of polyphenols. In general quercetin which reduced the fluorescence exhibited slightly stronger quenching effect in the presence of AMC than in the absence of it. On the contrary compounds capable to increase the fluorescence (6-MF, urolithin A and B) had weaker effects in the presence of AMC than in the absence of it. For instance, the fluorescence increases caused by urolithin A in serum were statistically significant without AMC, but slightly smaller and not statistically significant in its presence (Table 1.3).

Temperature exposures had an influence on the most of the polyphenols' fluorescent interactions as well. In the most cases the incubation at 37 °C tended slightly to weaken the quenching effects of quercetin. Temperature exposures decreased the effects of urolithin B in serum. Elevated temperature augmented the effects of 6-MF in assay buffer and DPP IV solution, but only in the absence of AMC (comparison 0 min and 30 min). The described observations might be due to the instability of the compounds.

Particular attention should be paid when using fluorescence based screening assays because small molecules of interest might interact with the experimental set-ups and result in misleading outcomes [412]. Such interactions of natural compounds with enzyme inhibition assays had already been described [413]. The authors claimed that intrinsic fluorescence of the compounds had a greater impact on the assay readouts than the quenching which seems to be in accordance with our results. The present data confirms the finding in the cited study that quercetin can quench the fluorescence and

alter the study outcome. Anyway, in the present approach this compound was tested at much lower concentration (5 µM) compared to the previous investigation (100 µM) and the statistical significance of this effect was confirmed.

To the best of our knowledge, the intrinsic fluorescence of urolithins in serum had not been described so far. The similarity of the chemical structures of urolithins and AMC might explain these effects to some extent (Figure 38).



AMC **Urolithin B**

Figure 38: Chemical structures of the fluorescent product AMC (7-aminomethyl-4-methylcoumarin) and one of the investigated compounds – intestinal metabolite of ellagic acid urolithin B, which interfered with the fluorescent assay.

As the interest in the bioactivities of intestinal metabolites is increasing [414], this artefact should be considered in *in vitro* DPP IV inhibition studies and other fluorescent based studies employing similar conditions.

4.4 Conclusions: Fluorescence interactions

Polyphenols may interact with DPP IV inhibitor screening assay and their properties should be considered prior to investigation. The compounds that quench fluorescence (quercetin) can be a reason for falsely positive results, and the compounds that increase the fluorescence (urolithin B, 6MF) can mask their own activity or those of other compounds if investigated in a more complex mixture.

C Experimental Setup

1 Effects of Pycnogenol® on active GLP-1, DPP IV, relaxin-2 and adiponectin

1.1 Clinical study

The Ethical committee of the Medical School, Bratislava, Slovak Republic, approved the study, and all of the participants had given written informed consent. The volunteers were asked to keep a regular diet without additional antioxidants. Dietary sources of polyphenols, such as tea, coffee, fruit juice, marmalade were temporary avoided during the whole course of the study.

1.2 Chemicals and reagents

Sitagliptin	Sigma-Aldrich, USA
Liquid Nitrogen	Tyczka Industrie-Gase GmbH, Würzburg

1.2.1 Specific chemicals and reagents for ELISA measurements

Glucagon-Like Peptide-1 (active) ELISA Kit	EMD Millipore, Missouri, USA
Human DPP IV/CD26 ELISA Kit	Sigma-Aldrich, USA
Human Relaxin-2 Quantikine ELISA Kit	R&D Systems, Inc., USA
Human Total Adiponectin/Acrp30 Quantikine ELISA Kit	

1.2.2 Specific chemicals and reagents for DPP IV serum enzymatic activity assay

DPP IV Activity Assay Kit	Sigma-Aldrich, USA

1.3 Materials and devices

Sterile vials of 2 mL	Sarstedt AG & Co., Nümbrecht, Germany
Sterile tubes 15, 50 mL	
Rack for tubes 15 mL	
Plate shaker, Type Mini-Rocker, 200 x 200 mm	Hartenstein GmbH, Würzburg, Germany
Polystyrene storage box	
Cryobox	
Eppendorf Research® Plus micropipettes: 0.5 – 10 µL; 10 – 100 µL; 100 – 1000 µL	Eppendorf AG, Germany
Eppendorf Research® 8-channel Pipette	
Megafuge 1.0 R	Thermo Fisher Scientific, Waltham, MA, USA
Multiskan Ascent, Plate-reader, filter 450 nm	
Ascent Software, Version 2.6	
HeraCell 240i, CO_2-Incubator	
Vortex Reax 1, Type 54111	Heidolph Instruments, Germany
Unimax 1010, Horizontal Shaker	
Plate sealer, Easy seal sealer clear, 80 x 140 mm 676001	Greiner Bio-One International, Austria
Ice generator	ZIEGRA Eismachinen GmbH, Germany
Microfuge 22R Centrifuge	Beckman Coulter GmbH, Krefeld

1.3.1 Specific materials und devices for ELISA measurements

PerkinElmer Luminescence Spectrometer LS 50 B	PerkinElmer Inc., USA
FL WinLab™	

1.4 Serum levels of GLP-1, DPP IV, relaxin-2, adiponectin. Serum enzymatic activities of DPP IV

For detection of target molecules' levels and activities in human serum respective commercially-available "sandwich"-principle ELISA (C-1.2.1) and enzymatic activity assay (C-1.2.2) kits were used.

1.4.1 GLP-1 (active) serum concentrations

The intact GLP-1 levels in serum samples were analyzed by the Glucagon-like Peptide-1 (active) ELISA Kit which was able to specifically detect the active forms of GLP-1: GLP-1 (7-36 amide) and GLP-1 (7-37). The black 96-well plate was covered with capture monoclonal antibody that bound specifically the N-terminal of GLP-1 and therefore recognized only the active forms of the peptide. The detection antibody was an anti-GLP-1-alkaline phosphatase conjugate binding the immobilized GLP-1. Due to its enzymatic activity this conjugate was able to be quantified by addition of MUP (4-methylumbelliferyl phosphate). Alkaline phosphatase cleaved the phosphate residue and released the fluorescent product umbelliferone and its fluorescence was measured at excitation/emission 355 nm/460 nm with the fluorescence plate reader (C-1.3.1). As the fluorescent signal was directly proportional to the concentration of active GLP-1 in the investigated sample, it was calculated via standard curves of reference standards provided with the kit. They contained known concentrations of GLP-1 (7-36) diluted in assay buffer. Standard curves were obtained within each experimental approach (Figure 39).

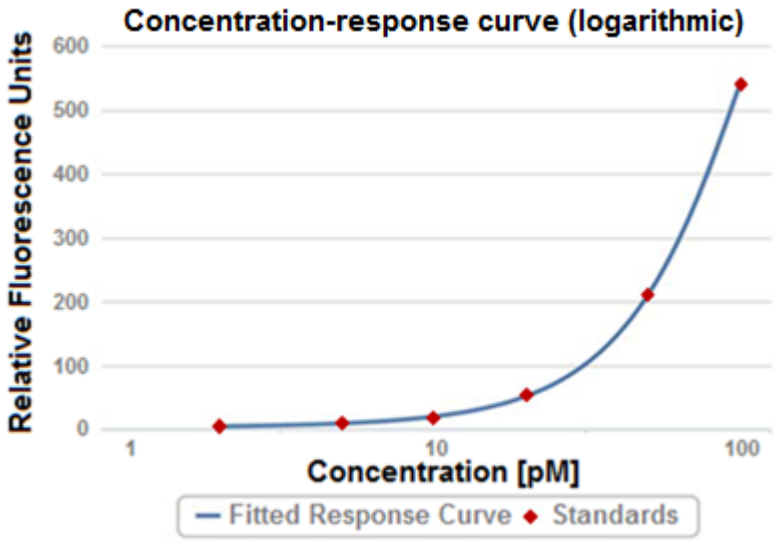


Figure 39: Representative standard curve for the GLP-1 (active) ELISA. The data was analyzed with free online ELISA Software [415]. The curve was fitted using polynomial cubic regression algorithm. On the x-axis the logarithmic values of GLP-1 concentrations (in pM) were plotted and on the y-axis the relative fluorescence values (RFU) after subtraction of the experimental blank. The fluorescence was measured at the excitation/emission wavelength of 355 nm/460 nm. The average accuracy of the standard curve was 99.96 %. The unknown sample concentrations were calculated from their relative fluorescence values in regard with the standard curve.

Serum samples were investigated undiluted. They were thawed at room temperature and were gently vortexed and shaken. Afterwards, they were aliquoted and spiked with 100 nM sitagliptin (final concentration). The addition of a DPP IV inhibitor and cooling the samples in an ice bath for prevention of enzymatic degradation of the active GLP-1 forms had previously been suggested [416]. Subsequently the assay was performed according to the manufacturer's protocol with one deviation. Apart from the original protocol the plate was incubated with assay buffer for 30 minutes before the addition of reference standards and the investigated samples in order to block the unspecific binding sites. Quality controls (QC1= 5.1 – 11 pM and QC2= 30 – 63 pM) were added to the plate randomly between the wells with samples. Assay characteristics of the Glucagon-like Peptide-1 (active) ELISA Kit are listed in Table 10.

Table 10.: Assay Characteristics of the Glucagon-Like Peptide-1 (active) ELISA Kit according to the manufacturer:

Sensitivity	Limit of detection: 2 pM (by 100 µL sample size)
Specificity	100 % for the active forms: GLP-1 (7-36 amide) and GLP-1 (7-37). Cross–reactivity with other forms of GLP-1 [such as GLP-1 (9-36 amide)], GLP-2 or glucagon has not been detected
Intra-Assay Variation	6 – 9 % (by concentration range 4 – 76 pM)
Inter-Assay Variation	< 1 – 13 % (by concentration range 4 – 76 pM)

1.4.2 DPP IV serum concentrations

DPP IV serum concentrations were analyzed by the Human DPPIV/CD26 ELISA Kit which was an indirect "sandwich" ELISA. Briefly, the antibody against human DPP IV covering the wells captured and immobilized DPP IV-molecules from serum samples which afterwards were recognized by the biotinylated secondary antibody. Antibody-linked biotin bound to streptavidin-horseradish peroxidase (HRP) conjugate with high specificity and affinity [417]. The latter enzyme catalyzed color reaction which was terminated by addition of acid stop solution. The optical density (O.D.) was immediately determined by a colorimetric detection at 450 nm.

All samples were diluted 500- to 750-fold to fall into the dynamic range of the assay (32.77 – 8000 pg/mL). All samples from individual participants were measured during the same experimental approach to avoid the inter-assay variations. The assay was performed according to the standard protocol provided by the manufacturer. As the O.D. was directly proportional to the concentration of human DPP IV in the investigated sample, it was calculated through standard curves of reference standards with known concentrations obtained by dilution of the recombinant human DPP IV/CD26 protein from the kit in assay

buffer before use. All of the standard curves were fitted using 5-parameter logistic (5-PL) regression (Figure 40). Assay characteristics of the Human DPPIV/CD26 ELISA Kit are shown in Table 11.

Table 11.: Characteristics of the Human DPPIV/CD26 ELISA Kit according to the manufacturer:

Sensitivity	Limit of detection: 25 pg/mL (by 100 µL sample size)
Specificity	The ELISA kit showed no cross-reactivity with 56 diverse cytokines
Intra-Assay Variation	< 10 %
Inter-Assay Variation	< 12 %

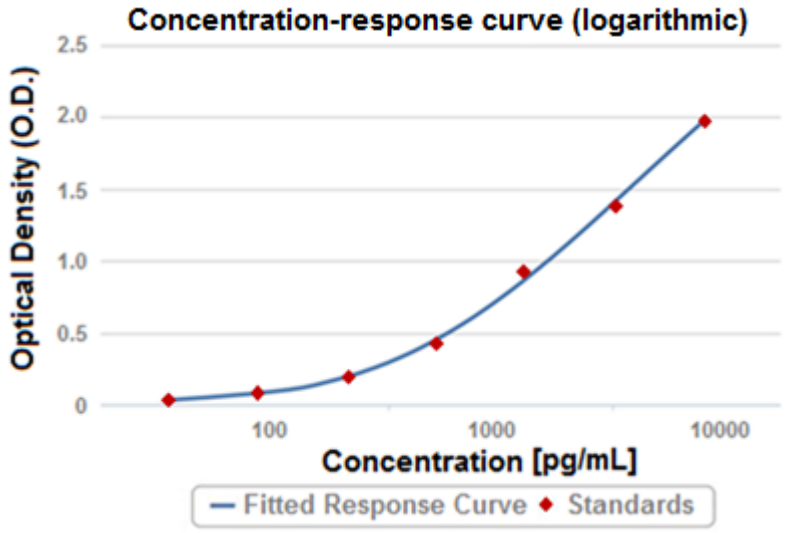


Figure 40: Representative standard curve for the Human DPP IV/CD26 ELISA. The data was analyzed with free online ELISA Software [415]. The curve was fitted using 5-parameter logistic regression. On the x-axis the logarithmic values of DPP IV concentrations (in pg/mL) were plotted and on the y-axis the optical density values (O.D.) after subtraction of the experimental blank. The absorbance was measured at the wavelength of 450 nm. The average accuracy of the standard curve was 102.4 %. The unknown sample concentrations were calculated from their optical density values in regard with the standard curve.

1.4.3 Relaxin-2 serum concentrations

The levels of relaxin-2 in the volunteers' samples were investigated by means of the Human Relaxin-2 Quantikine ELISA Kit – a "sandwich" immunoassay, which was one of the most sensitive and specific (utilizing monoclonal antibody) available. The principle of this ELISA kit was similar to the Human DPPIV/CD26 ELISA Kit. The most significant difference was that instead of streptavidin-biotin system the assay utilized a detection polyclonal antibody for relaxin-2 directly linked to the reporter-enzyme (HRP). Similarly, the O.D. was measured at 450 nm.

All of the samples were investigated undiluted. The assay was performed according to the standard protocol provided by the manufacturer. The dynamic range of the immunoassay was 7.8 – 500 pg/mL. Human recombinant relaxin-2 from the kit was diluted in assay buffer and reference standards were acquired. Standard curves were fitted using 5-parameter logistic (5-PL) regression (Figure 41). Assay characteristics of the Human Relaxin-2 Quantikine ELISA Kit are indicated in Table 12.

Table 12.: Characteristics of the Human Relaxin-2 Quantikine ELISA Kit according to the producer:

Sensitivity	The minimum detectable concentration of relaxin-2 ranged from 0.26 to 4.57 pg/mL; the mean minimal detectable concentration was 1 pg/mL
Specificity	The assay recognized natural and recombinant human relaxin-2. No significant cross reactivity with related molecules such as relaxin-1, relaxin-3, and insulin has been observed
Intra-Assay Variation	2.3 – 4.7 % (by concentration range 34.4 – 206 pg/mL)
Inter-Assay Variation	5.5 – 10.2 % (by concentration range 40.8 – 220 pg/mL)

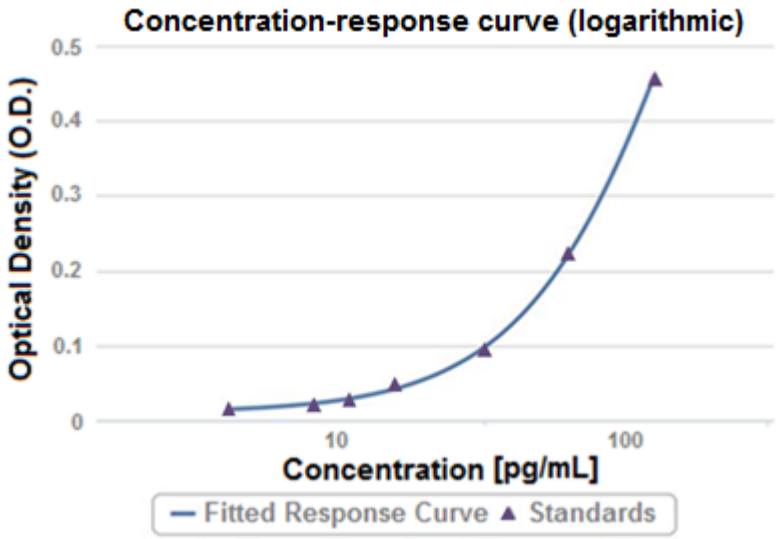


Figure 41: Representative standard curve for the Human Relaxin-2 ELISA. The data was analyzed with free online ELISA Software [415]. The curve was fitted using 5-parameter logistic regression. On the x-axis the logarithmic values of relaxin-2 concentrations (in pg/mL) were plotted and on the y-axis the optical density values (O.D.) after subtraction of the experimental blank. The absorbance was measured at the wavelength of 450 nm. The average accuracy of the standard curve was 99.2 %. The unknown sample concentrations were calculated from their optical density values in regard with the standard curve.

1.4.4 Total adiponectin serum concentrations

Total adiponectin concentrations in serum samples were investigated using the Human Total Adiponectin/Acrp30 Quantikine ELISA Kit. The principle of this assay and the experimental procedures were analogical to the Human Relaxin-2 Quantikine ELISA Kit's ones described above. All of the samples were diluted 1:200 to fall into its dynamic range (3.9 – 250 ng/mL). Serial dilutions of human recombinant adiponectin in assay buffer were used as standards. The standard curves were defined individually for every experimental approach and were fitted either by 4-parameter or by 5-parameter logistic regression (Figure 42). Assay characteristics of the Human Total Adiponectin/Acrp30 Quantikine ELISA Kit are shown in Table 13.

Table 13.: Characteristics of the Human Total Adiponectin/Acrp30 Quantikine ELISA Kit according to the manufacturer:

Sensitivity	The minimum detectable concentration of adiponectin ranged from 0.079 to 0.891 ng/mL; the mean minimal detectable concentration was 0.246 ng/mL
Specificity	The assay recognized natural and recombinant human adiponectin. No significant cross reactivity with related molecules such as TNF-family members has been observed
Intra-Assay Variation	2.5 – 4.7 % (by concentration range 19.8 – 143 ng/mL)
Inter-Assay Variation	6.8 – 6.9 % (by concentration range 20.5 – 157 ng/mL)

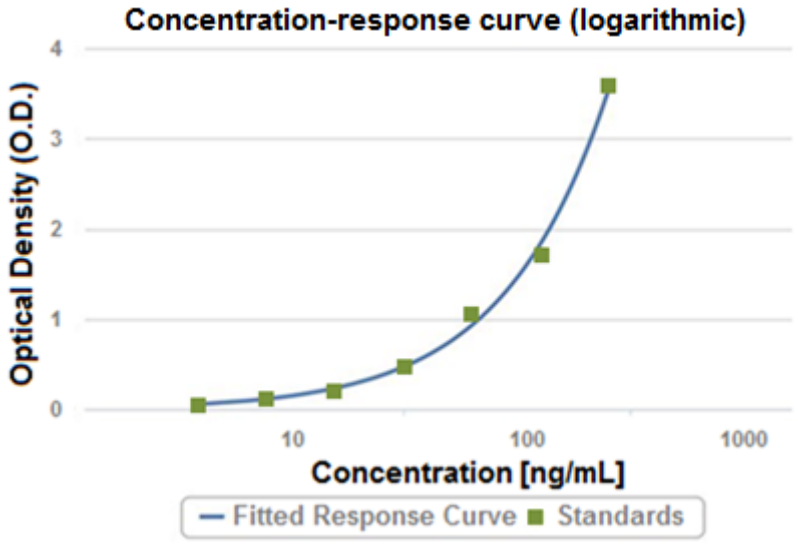


Figure 42: Representative standard curve for the Human Total Adiponectin ELISA. The data was analyzed with free online ELISA Software [415]. Standard curves were fitted using 4- or 5-parameter logistic regression. On the x-axis the logarithmic values of total adiponectin concentrations (in ng/mL) were plotted and on the y-axis the optical density values (O.D.) after subtraction of the experimental blank. The absorbance was measured at the wavelength of 450 nm. The average accuracy of the indicated standard curve was 98.5 %. The unknown sample concentrations were calculated from their optical density values in regard with the standard curve.

1.4.5 DPP IV serum enzymatic activities

DPP IV enzyme activities of volunteers' serum samples were measured directly and kinetically using the DPP IV Activity Assay Kit (C-1.2.2), which utilized a non-fluorescent substrate (H-Gly-Pro-AMC) that underwent proteolysis by DPP IV and released the fluorescent product AMC (7-amino-4-methyl coumarin), which was measured (λ_{ex} = 360 nm/ λ_{em} = 460 nm). The assay was based on Fujiwara and Tsuru method (1978) [418]. Along with the measured samples, respective sample blanks of serum were obtained by addition of sitagliptin (100 nM) specifically inhibiting the intrinsic DPP IV serum activity and considering the respective matrix effect and rest activities of enzymes mimicking DPP IV. To quantify the released AMC, a 1 mM AMC standard solution was used to generate standard concentrations of 0 - 100 pmol/well AMC (Figure 43). The measured activities of the investigated serum samples ranged from 476.36 µU/mL to 1212 µU/mL.

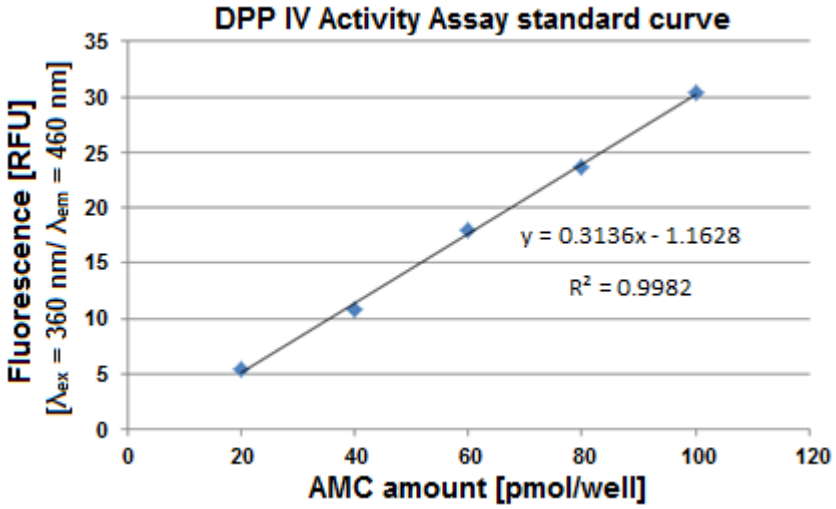


Figure 43: Representative standard curve for the DPP IV Activity Assay Kit. The data was analyzed with Microsoft Excel® Version 2010 (Microsoft Corporation, Redmond, USA). The curve was fitted using linear regression. On the x-axis was plotted the AMC amount (pmol/well) and on the y-axis – the relative fluorescence [relative fluorescence units (RFU)] after subtraction of the experimental blank. The fluorescence was measured at λ_{ex} = 360 nm/ λ_{em} = 460 nm. The average accuracy of the standard curve was 100.5 %. The amount of AMC generated by DPP IV for defined period of time in investigated samples was calculated from the relative fluorescence values in regard with the standard curve.

The assay was performed according to the manufacturer's protocol. Briefly, all serum samples were diluted 1:10, excluding the probes of participant 20, which were diluted 1:20, due to presence of hemolysis. Equal volumes of the diluted samples were directly added to the wells. Purified DPP IV solution provided with the kit served as a positive control. After 10 minutes incubation of the plate (37 °C, shaking) the master reaction mix (diluted the

DPP IV substrate in assay buffer) was added to each sample's and sample blank's well, but not to the standards' wells. The plate was protected from light and incubated at 37 °C on horizontal shaker. After 5 minutes the initial fluorescence measurement was performed. Afterwards, the plate was incubated further and a kinetic measurement was performed every 5 minutes, usually up to 20 minutes. Practically, the measurements continued until the most active sample exceeded the fluorescence value of the highest standard (100 pmol/well). Typically, the penultimate measurement (15 min) was used as a final one. The differences in fluorescence (FLU) between the initial ($T_{initial}$) and the final (T_{final}) time point were determined for every sample and sample blank:

$$\Delta FLU = (FLU)_{final} - (FLU)_{initial}$$

The sample blank value ($\Delta FLU_{sample\ blank}$) was subtracted from the sample value (ΔFLU_{sample}):

$$\Delta FLU' = \Delta FLU_{sample} - \Delta FLU_{sample\ blank}$$

The amount of AMC (B) released by the DPP IV in each sample between $T_{initial}$ and T_{final} was determined by $\Delta FLU'$ using a standard curve (Figure 43).

The DPP IV activity of each sample was determined as following:

$$DPP\ IV\ Activity = \frac{B \times (Sample\ Dilution\ Factor)}{(Reaction\ Time) \times V}$$, where:

B represents the amount of AMC released between the initial ($T_{initial}$) and the final (T_{final}) time point [pmol];

Sample dilution factor represents dilution of the serum sample. Typically, its value was 10 (or 20 for participant No. 20);

Reaction time is the time period between the initial and the final time point: $Reaction\ time = T_{final} - T_{initial}$ [min];

V is the sample volume added to well [mL];

DPP IV Activity is calculated in the following units: [pmol/min/mL] = [µU/mL];

1.5 Statistical analysis

1.5.1 Standard curve fitting

The fitting of standard curves was performed using linear [Microsoft Excel® Version 2010 (Microsoft Corporation, Redmond, USA)] or non-linear regression (4-PL, 5-PL, polynomial cubic regression) as calculated by the free online tool for analysis of ELISA (Elisaanalysis.com, Crux Biolab Pty Ltd., Australia) [415] as indicated above.

1.5.2 Descriptive statistics and normality

The mean values, standard deviations (S.D.), medians, and mean deviation (Mean Dev.) were calculated with Microsoft Excel® Version 2010. The normality of the distribution of the data was checked by the Shapiro-Wilk test [419], using the freely available Real Statistics Add-in© (2013-2016, Charles Zaiontz) of Excel [420].

1.5.3 Outliers detection and exclusion

Values bigger than 2.5 S.D. were identified as outliers by the Box Plot test [191]. Additionally, outliers were detected by the two-sided Grubbs' test [192] which defined outliers as values meeting the following requirement:

$$G > G_{crit}, \text{ where}$$

$G = \frac{\max|x_i - \bar{x}|}{S.D.}$ and G_{crit} can be calculated as following:

$$G_{crit} = \frac{(N-1)}{\sqrt{N}} \sqrt{\frac{(t_{\alpha/(2N),N-2})^2}{N-2+(t_{\alpha/(2N),N-2})^2}}, \text{ where}$$

$t_{\alpha/(2N),N-2}$ is the critical value of the *t*-distribution, with *N-2* degrees of freedom and a significance level of $\alpha/(2N)$.

For this purpose the Real Statistics Add-in© (2013-2016, Charles Zaiontz) of Excel was used [420].

1.5.4 Statistical significance

The statistical significance of the differences between DPP IV concentrations and activities of control and treated state was examined using a two tailed paired Student's t-test. The statistical significance of the differences between GLP-1 (active) and total adiponectin serum levels were investigated by one-way ANOVA. The significance level α was set to be 0.05 for all statistical significance tests performed. The tests were performed with the Real Statistics Add-in© (2013-2016, Charles Zaiontz) of Excel [420]. Alternatively, the freely available online statistical web calculator Astatsa (2016, Navendu Vasavada) performing

Tukey HSD (honestly significant difference) test for multiple group comparison was used [421].

2 Effects of polyphenols on protein phosphorylation

2.1 Cells, chemicals and reagents

2.1.1 Cells used in *in vitro* models

Ea.hy926 is a hybrid cell line, which had been derived by fusing human umbilical vein endothelial cells (HUVEC) with the permanent lung epithelial cell line A549 and it was generously provided for use from Dr. C.J. Edgell (University of North Carolina, NC, USA) [422]. Frozen aliquots with low-passage mycoplasma-negative cells were obtained from the Biocenter of the University of Würzburg, Department of Microbiology. Ea.hy926 cells were generally used between passages 6 and 30.

HUVEC are primary endothelial cells, which were purchased from Thermo Fisher Scientific (catalog number: C01510C). According to the manufacturer they were obtained and pooled from multiple donor isolates. These cells were used between passages 3 and 6.

Cells underwent regularly tests for mycoplasma contamination (C-2.4.6).

2.1.2 Specific chemicals and reagents for cell culture

2.1.2.1 Cell culture media

Dulbecco's modified essential medium, high glucose (HG DMEM, 4500 mg/L glucose)	Sigma-Aldrich, USA
Dulbecco's modified essential medium, low glucose (LG DMEM, 1000 mg/L glucose)	
M199 without phenol red (M3769)	

Both DMEM formulations with high and low glucose content were phenol red-free, because this compound has been described to possess some estrogen-like properties [423, 424].

DMEM growth media was supplemented as following (final concentrations):

- 3.7 g/L $NaHCO_3$
- 10 % heat inactivated fetal bovine serum (FBS)
- 2 mM L-glutamine
- 1 mM non-essential amino acids (NEA)
- 1 mM sodium pyruvate
- mixture of 100 U/mL penicillin and 100 µg/mL streptomycin (1 % Pen/Strep)

Similarly, M199 was used after the following supplementation:

- 2.2 g/L $NaHCO_3$
- 20 % heat inactivated fetal bovine serum (FBS)
- 2 mM L-glutamine
- mixture of 100 U/mL penicillin and 100 µg/mL streptomycin (1 % Pen/Strep)
- 1 % low serum growth supplement (LSGS)

The glucose content of this medium was 1000 mg/L, corresponding to the control conditions.

2.1.2.2 Additional chemicals and reagents for cell culture

Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich, USA
Trypsin/EDTA solution 1x (trypsin 0.05 %/EDTA 0.02 %)	
Sodium pyruvate solution (100 mM)	
Non-essential amino acids	
Insulin solution human (10 mg/mL)	
Dimethyl sulfoxide (DMSO)	
Protease Inhibitor cocktail (for mammalian cells and tissue extracts, P8340)	
CelLytic™ M lysis buffer	
RIPA lysis buffer	
Gelatin from porcine skin	
L-Glutamine (200 mM)	Biochrom AG, Berlin
Penicillin-Streptomycin (10000 IU/mL)	
Trypan blue solution 0.5 % (m/V)	
Fetal bovine serum (FBS) – heat inactivated	
Low serum growth supplement (LSGS)	Life Technologies, USA
PhosSTOP™	Roche Diagnostics GmbH, Germany

Histamine dihydrochloride	Fluka BioChemika, Switzerland
Histamine base	
Millipore water	Water processing system, Millipore, Schwalbach, Germany
Isopropanol	Inventory at the Faculty of Chemistry and Pharmacy (University of Würzburg)

2.1.2.3 Polyphenolic compounds

Stock solutions (10^{-2} M) of the investigated polyphenols in DMSO were prepared and stored at – 80 °C. Compounds and their purities are indicated below:

Compound	Purity (%)	Producer	Compound	Purity (%)	Producer
Resveratrol	> 98	Tokyo Chemical Industry (TCI) Co., Ltd., Japan	7,8-Dihydroxy-flavone	> 98	Tokyo Chemical Industry (TCI) Co., Ltd., Japan
Pinostilbene	> 97				
Pterostilbene,	> 98		(-)-Epigallocatechin gallate	> 98	
3,4',5-Trimethoxy-trans-stilbene	> 96				
			Flavanone	> 98	
Piceatannol	> 98		Urolithin A	N/A	Newchem Technologies Ltd, UK
Oxyresveratrol	> 95		Urolithin C	N/A	
Naringenin	> 93		Urolithin D	N/A	
Apigenin	> 98		Urolithin B	≥ 95	Sigma-Aldrich, USA
Taxifolin	≥ 85		Flavone	≥ 99	
Genistein	> 96		Chlorogenic acid	> 95	
3-Hydroxyflavone	> 98		Morin	> 85	
3-Methoxyflavone	> 98		Quercetin	> 98	
7-Methoxyflavone	> 98		Caffeic acid	≥ 98	
3,4'-Dihydroxy-flavone	> 97		(+)-Catechin	> 99	
			Ellagic acid	> 95	
3-Hydroxy-4'-methoxyflavone	> 98		Luteolin	≥ 98	Aladdin, Shanghai, China
			Trans-ferulic	> 99	
Kaempferol	> 97		(-)-Epicatechin	≥ 98	
Myricetin	> 97		Baicalin	> 95	
Chrysin	> 97		Wogonoside	≥ 98	
Fisetin	> 96		(-)-Gallocatechin gallate	≥ 98	
Baicalein	> 98				
6-Hydroxyflavone	> 98		(-)-Epigalocatechin	≥ 98	
6-Methoxyflavone	> 98		Vitexin	≥ 98	TAUTO®, China

Catechin metabolites M1 [δ-(3,4-dihydroxyphenyl)-γ-valerolactone] and M2 [δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone] were synthesized by M. Rappold and kindly provided for use. Accordingly, stock solutions (10^{-2} M) were employed for cell culture experiments.

2.1.3 Specific chemicals and reagents for Western blot analysis

2.1.3.1 Chemicals and reagents for SDS PAGE and protein transfer (electroblotting), blocking, and staining

Sodium dodecyl sulfate	Inventory at the Faculty of Chemistry and Pharmacy (University of Würzburg)
Glycine	
Methanol	
Sodium chloride	
Rotiphorese Gel 30 (37.5:1) (acrylamide-bisacrylamide mixture)	Carl Roth GmbH + Co, Karlsruhe, Germany
Ammonium persulfate (APS)	
TEMED	
Ponceau S	
Trizma® Base (TRIS)	Sigma-Aldrich, USA
Tween® 20	
TruPage™ DTT sample reducer 10x	
Nitrocellulose blotting membrane (Amersham™ Protran™ Supported 0.2 µm)	
Low-fat dry milk powder	J.M. Gabler – Saliter Milchwerk GmbH & Co. KG, Germany
Protein-Marker V Peqlab	VWR Peqlab, USA
Coomassie brilliant blue R-250	Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany
Bovine serum albumin (BSA)	GERBU Biotechnik GmbH, Heidelberg, Germany
Blotting paper (0.35 mm, medium absorbency)	Hartenstein GmbH, Würzburg, Germany

2.1.3.2 Antibodies used for Western blot analysis (Immunodetection)

All of the antibodies used for Western blot were purchased from Cell Signaling Technology, Inc., USA and are listed below:

Antibody's name	Cat. #
Phospho-Akt (Ser473) Antibody	9271
Akt Antibody	9272
Phospho-Akt (Thr308) 224F9 mAb	4056
Phospho-AMPKα (Thr172) (40H9) Rabbit mAb	2535
β-Actin (D6A8) Rabbit mAb	8457
Anti-rabbit IgG, HRP-linked Antibody	7074

2.1.3.3 Chemicals and reagents for Western blot development

Clarity™ Western ECL substrate	Bio-Rad Laboratones, Inc., USA

2.1.4 Specific chemicals and reagents for ELISA measurements

RayBio® Human/Mause/Rat Phospho-Akt (S473) and Total Akt ELISA Kit	Raybiotech, Inc., USA

2.1.5 Specific chemicals and reagents for BCA-Assay for protein concentration

Pierce® BCA Protein Assay Kit (bicinchoninic acid assay)	Thermo Fisher Scientific, Waltham, MA, USA

2.1.6 Specific chemicals and reagents for mycoplasma test

LookOut® Mycoplasma PCR Detection Kit	Sigma-Aldrich, USA
JumpStart Taq DNA Polymerase	
Agarose	
DAPI (4',6-diamidino-2-phenylindole)	

2.2 Materials and devices

PL300 Balance	Mettler-Toledo GmbH, Giessen, Germany
AB204-S/PH Fine balance	
Ultrasonic bath, USR 30	Merck eurolab, Darmstadt, Germany
Thermomixer compact	Eppendorf AG, Germany

All routinely used vials and tubes for sample preparation, handling, storage, and experimental procedures were purchased from Sarstedt AG & Co., Nümbrecht, Germany, if nothing else was specially indicated.

2.2.1 Specific materials and devices for cell culture

HeraSafe KS-12 Class II, laminar flow biohazard hood	Thermo Fisher Scientific, Waltham, MA, USA
Heracell, 240 I, cell culture incubator	
Mr. Frosty™ freezing container	
Eclipse TS 100, inverted microscope	Nikon GmbH, Düsseldorf, Germany
Cell culture flasks, T-75, polystyrene (83.3911.002)	Sarstedt AG & Co., Nümbrecht, Germany
Cell culture plates, (83.3920), 6-, 12-, 96-well	
Serological pipettes 10 ml (86.1254.001)	
Serological pipettes 25 ml (86.1685.001)	
Counting chambers, C-Chip™ disposal, type "Neubauer Improved"	Hartenstein GmbH, Würzburg, Germany
Suction device for cell culture/vacuum pump	
Accu jet® pro pipette controller	BRAND GMBH + CO KG
Cell counter (Desk Tally Counter)	ENM, England
Cell scraper 240 mm	TPP AG, Switzerland

2.2.2 Specific materials and devices for Western blot analysis

Mini-PROTEAN Tetra®, Gel-Electrophoresis Equipment	Bio-Rad Laboratones, Inc., USA
Mini-Trans Blot® cell (wet-transfer system)	
Mini-Protean® Plates (glass)	
FluorChem FC2 Doku System	Alpha Innotech GmbH, Germany
FluorChem FC2 Software	
ImageJ JAVA Software	National Institutes of Health, USA
Electrophoresis power supply EPS 301	Amersham Pharmacia Biotech Inc., USA

2.2.3 Specific materials and devices for ELISA measurements

ELISA plates were read-out by Multiskan Ascent Plate-reader (C-1.3) at the wavelength of 450 nm.

2.2.4 Specific materials and devices for protein determination

UVmini 1240 (UV meter)	SHIMADZU Corp., Kyoto, Japan
UV cuvettes (67.759)	Sarstedt AG & Co., Nümbrecht, Germany

2.3 Frequently used solutions

2.3.1 Solutions used for sample preparation and Western blot analysis

Buffer/Solution	Substance	Final concentration	Quantity
Loading buffer (4 x Laemmli without β-mercaptoethanol)	Glycerol (100 %)	40 % (v/v)	4.0 mL
	TRIS 1M (pH = 6.8)	240 mM	2.4 mL
	SDS	8 % (w/v)	0.8 g
	Bromphenol blue	0.04 % (w/v)	4.0 mg
	Millipore water		3.1 mL
Running buffer (10 x SDS buffer) (pH= 8.3)	TRIS base	250 mM	30.3 g
	Glycin	1.92 M	144.0 g
	SDS	1 % (w/v)	10.0 g
	Millipore water		ad 1.0 L
Running buffer (1 x SDS buffer)	10 x SDS Buffer	10 % (v/v)	0.1 L
	Millipore water		ad 1.0 L
Transfer buffer (pH= 8.1 – 8.5)	TRIS base	20 mM	2.40 g
	Glycin	150 mM	11.24 g
	Methanol	20 % (v/v)	200.00 mL
	Millipore water		ad 1.0 L
Modified transfer buffer	0.037 % SDS in transfer buffer		
TRIS-Buffered Saline + Tween® 20 (TBST) (1 x Washing buffer) (pH= 7.6)	TRIS 1M (pH = 7.6)	20 mM	20.0 mL
	NaCl	150 mM	75.0 mL
	Tween® 20	0.05 % (v/v)	500.0 µL
	Millipore water		ad 1.0 L
Blocking buffer	5 % Low-fat dry milk in TBST (w/v)		
BSA buffer	5 % BSA in TBST (w/v)		
NaN_3 solution	10 % NaN_3 in Millipore water (w/v)		

2.3.2 Solutions used for polyacrylamide gel cast

APS solution (10 %)	10 % APS in water (w/v)
SDS solution (10 %)	10 % SDS in water (w/v)
TRIS (stacking gel)	1.5 M TRIS, pH= 6.8
TRIS (resolving gel)	1.5 M TRIS, pH= 8.8

2.3.3 Stripping solutions

Soft stripping buffer (pH= 2.2)	Glycin	200 mM	15.0 g
	SDS	0.1 % (w/v)	1.0 g
	Tween 20	1.0 % (v/v)	10.0 mL
	Millipore water		ad 1.0 L

2.3.4 Staining solutions

Coomassie Blue staining solution	Methanol	45.0 % (v/v)	450 mL
	Glacial acetic acid	10.0 % (v/v)	100 mL
	Coomassie blue R-250	0.25 % (w/v)	2.5 g
	Millipore water		ad 1.0 L
Ponceau S reversible staining solution	Ponceau S	0.1 % (w/v)	1.0 g
	Glacial acetic acid	5.0 % (v/v)	50 mL
	Millipore water		ad 1.0 L
Destaining Solution	Methanol	5.0 % (v/v)	50 mL
	Glacial acetic acid	7.5 % (v/v)	75 mL
	Millipore water		ad 1.0 L

2.3.5 Coating solution for cell culture flasks (HUVEC)

Gelatin Solution 1 % (autoclaved)	Gelatin from porcine skin	1.0 % (w/v)	1.0 g
	Millipore water		ad 0.1 L

2.3.6 Solutions for mycoplasma test

10 x TBE (pH= 8.4)	Tris	890 mM	108.0 g
	Boric acid	890 mM	55.0 g
	Na_2EDTA	25 mM	9.3 g
	Millipore water		ad 0.5 L
1 x TBE	10 x TBE	10 % (v/v)	0.1 L
	Millipore water		ad 1.0 L
DAPI stock solution	DAPI	14.3 mM	5 mg
	Millipore water		ad 1.0 mL

2.4 Experimental design

2.4.1 *In vitro* cell culture model – routine methods

2.4.1.1 Cultivation, subcultivation, and medium change

Immortalized human endothelial cell line Ea.hy926 was cultured according to a standard protocol [425] in phenol red-free DMEM cell culture medium with different glucose content [low glucose (LG) – 1000 mg/L, high glucose (HG) – 4500 mg/L] supplemented as indicated above (C-2.1.2.1). Briefly, cells were seeded into 75 cm^2 flasks at a density of 2000 – 2500 cells/cm^2. The medium was changed every second day. When cells reached 90 % confluence they were passaged. For this purpose, they were rinsed three times with warm PBS and treated with trypsin. Afterwards, detached cells were resuspended in a fresh warm medium. Cell suspension was either seeded into flasks for further cultivation or into 6-well plates at a density of 0.3 x 10^6 cells per well for experiments.

Primary endothelial cells HUVEC were cultivated according to the protocol described above with some deviations. Instead of DMEM, M 199 supplemented as indicated in C-2.1.2.1 was used. All flasks and wells used for HUVECs were coated with a sterile 1 % gelatin solution. After trypsinization cells were mixed with growth medium and pelleted by centrifugation (7 min, 25 °C 1200 g) in order to remove the proteolytic enzyme. Then the cells were resuspended in a fresh warm M 199 and counted, passaged or seeded for an experiment as indicated.

2.4.1.2 Cellular cryopreservation and thawing

In order to cryopreserve endothelial cells, they were grown to confluence and treated with trypsin. They were suspended in a few milliliters of serum-supplemented DMEM in order to stop the action of the enzyme and then the cell suspension was centrifuged for 7 minutes at 25 °C, at 1200 g. The cell pellet was mixed with 4 mL fresh medium to obtain a cell suspension free of trypsin. Cells were counted and one part of the cell suspension was mixed with one part precooled freezing medium, containing 80 % FBS and 20 % DMSO. Thus, 0.5 – 1 x 10^6 cells pro vial were cryopreserved in medium containing 10 % DMSO and 45 % FBS (final concentration v/v). As such DMSO concentrations are cytotoxic at room temperature aliquots were put immediately into precooled freezing container (described under C-2.1.1) filled with isopropanol and was left at – 80 °C for 24 hours. This device ensured gradual cooling of cells at a rate of 1 °C per minute, which is optimal for their vitality. For long terms cell aliquots were stored in a liquid nitrogen tank (– 196 °C).

When needed, the cryopreserved cells were removed from the storage tank and the vial was warmed in a water bath (37 °C) under continuous shaking. When crystals almost disappeared, the cell suspension was gently mixed by a pipette tip and was transferred to a cell culture flask with warm (37 °C) growth medium. After 4 hours the majority of the cells was attached to the bottom of the flask and the medium was changed to remove residuals of DMSO. For HUVECs the rests of DMSO were removed through centrifugation and replacement with pre-warmed medium even before inoculation in the flask. This was crucial for their good viability.

2.4.1.3 Counting of the cells and vitality test

25 µL of the well-homogenized cell suspension obtained after trypsin treatment were either directly pipetted into the Neubauer chamber (for cell counting, C-2.2.1) or firstly mixed with trypan blue solution (for vitality test) at a ratio 1:1 and then pipetted into the Neubauer chamber. Cells from four fields of the chamber grid were counted under the microscope in a meandering manner, and upper and left edges of the fields were omitted to avoid the double counting. The total number of the cells was calculated as following:

$$\text{Number of cells/mL} = (\text{M}/4)\ \text{x F x } 10000, \text{ where}$$

M was the total number of cells counted,

F was the dilution factor: F= 1, for cell count without trypan blue; F= 2, for the vitality test.

Trypan blue staining was routinely performed to determine the vitality of cells. Intact cells were not permeable for the stain and remained brighter on a blue background. In contrast, dead cells were stained in dark blue, because of the impaired integrity of the cellular membrane and increased permeability of the stain.

2.4.1.4 Analysis of the effects of polyphenols on signaling pathways *in vitro*

After seeding cells were grown with 3 mL medium/well and left to reach confluence. Then they were serum deprived and thereby growth arrested for 18-20 hours. Afterwards, they were treated with the compounds of interest at a concentration of 10 µM for a defined time. Such polyphenol concentrations were considered as physiologically relevant [218, 275]. As a positive control was used histamine (known AMPK/eNOS-activatior) at a final concentration of 20 µM [426]. For Akt-activation insulin (1 µM) was utilized as a positive control [427] (Figure 44).

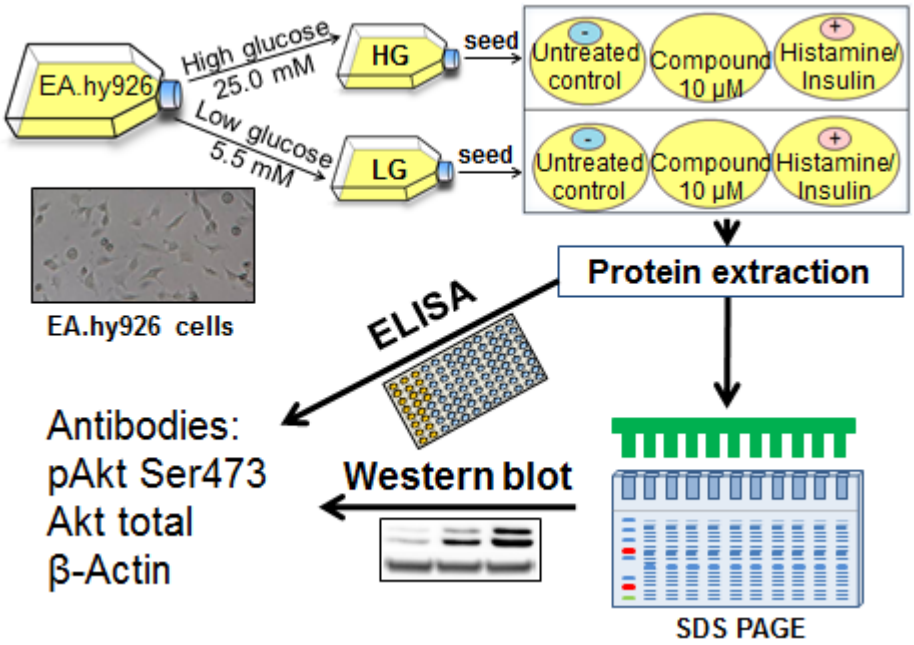


Figure 44: Flowchart of the experimental approach of analysis of the effects of polyphenols on signaling pathways in endothelial cells *in vitro*. Cells cultivated in HG (high glucose, 25.0 mM) and LG (low glucose, 5.5 mM) medium were seeded on 6-well plates. After reaching confluence, cells were treated with polyphenols and referent compounds, which served as positive controls. Cells were lysed and extracted proteins were separated by SDS PAGE and transferred to a nitrocellulose membrane. Proteins were detected immunologically by phosphospecific and total-protein antibodies. Alternatively, phosphorylation status of Akt was analyzed by quantitative ELISA.

Compounds revealing an inhibitory activity were investigated by a modified protocol. Individual polyphenols were applied on cells in standard DMEM with 10 % FBS from defined batch (1107A) in order to establish their potential to antagonize the growth factor-induced Akt-phosphorylation. As a positive control the cells from one well per plate were incubated in serum-free medium overnight. Absence of growth factors strongly decreased Akt-phosphorylation [428].

2.4.2 Cell lysis and sample preparation

After treatments cells were rinsed twice with ice-cold PBS and 300 µL precooled lysis buffer (CelLytic™ M or RIPA) supplemented with phosphatase and protease inhibitors were added to each well. Cellular proteins were extracted at 4 °C on a shaker for 15 minutes. Cells residues were scraped from the wells and the lysates were transferred to 1.5 mL precooled plastic tubes. They were centrifuged at 4 °C for 10 minutes at 14000 rpm

(rcf around 18000 g) in order to remove cellular debris. The supernatants were transferred into new tubes, vortexed, and briefly ultra-sonicated in order to homogenize obtained cell lysates. If needed, protein extracts underwent protein concentration determination (C-2.4.3). In order to reduce and denature proteins and thus to prepare samples for SDS PAGE and Western blot, Laemmli buffer (4X) without β-mercaptoethanol was added to the cellular extracts. Instead, DTT (Dithiothreitol) reducing agent (C-2.1.3.1) was added separately to the samples. They were incubated in the Thermomixer for 7 minutes at 70 °C under shaking (1000 rpm) and were vortexed and shortly spun. Then they were directly analyzed or stored at -20 °C. During the handling all of the samples were held on ice.

2.4.3 Determination of sample protein concentrations

Protein concentrations of cell lysates were quantified employing a bicinchoninic acid assay kit (BCA Assay, C-2.1.5). This approach utilized a modified colorimetric detection based on the method developed by Lowry [429]. The assay was performed according to manufacturer's instructions (test tube procedure, standard protocol) with some minor deviations. A blank and seven protein standards ranging from 62.5 to 2000 µg/mL were used. All investigated samples were diluted 1:3 with PBS. 30 µL of standards or diluted samples were mixed well with 600 µL working reagent (instead of 100 µL sample and 2 mL working reagent) and were incubated for 30 minutes at 37 °C. After cooling the absorbance of each blank, sample or standard was measured by UVmini 1240 spectrophotometer (C-2.2.4) at 562 nm. Unknown sample concentrations were determined by a standard curve obtained with every measurement (Figure 45). All samples were adjusted by addition of respective volume lysis buffer to them so that their protein concentrations were equal. Alternatively, the assay was carried out in 96-well plates according to manufacturer's protocol and absorbance was measured by Multiskan plate reader (C-1.3).

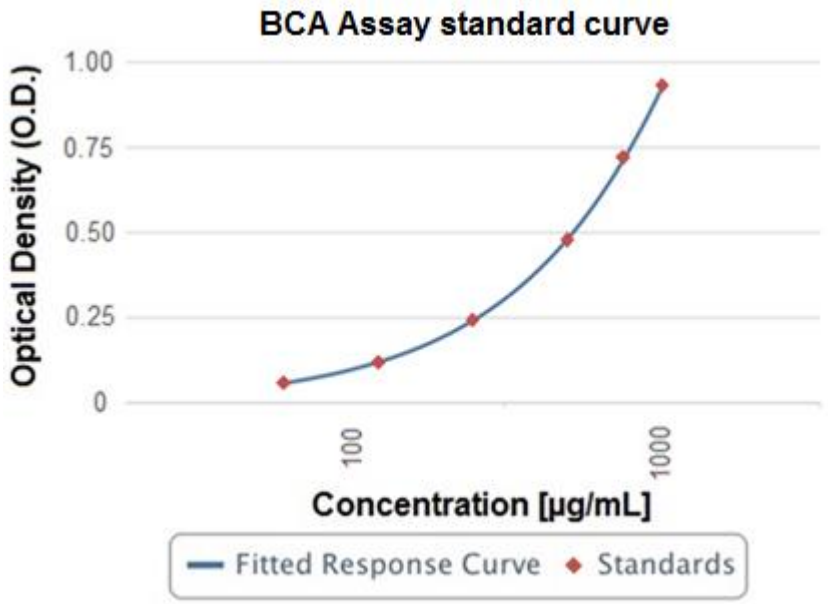


Figure 45: Representative standard curve of a BCA assay. The data was analyzed with free online regression analysis software [415]. The curve was fitted using 5-parameter logistic regression, indicating superiority over linear regression. On the x-axis the logarithmic values of standard protein concentrations (in µg/mL) were plotted and on the y-axis – the optical density values (O.D.) after subtraction of the experimental blank. The absorbance was measured at 562 nm. The average accuracy of the standard curve was 100.5 %. The unknown sample concentrations were calculated from their optical density values in regard with the standard curve.

2.4.4 Western blot analysis

Western blot is a widely used immunochemical method for protein analysis, firstly described by Burnette [430]. In the present work it was performed with phosphospecific antibodies against pAkt and (also pAMPK) (C-2.1.3.2). Total Coomassie gel staining (C-2.3.4), pan Akt, and actin beta (C-2.1.3.2) were used as loading controls. All of the reagents, materials and buffers needed for Western blot analysis have been described under C-2.1.3, C-2.2.2, and C-2.3.

2.4.4.1 SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

After reduction and denaturation (C-2.4.2), proteins were separated by SDS PAGE according to their molecular weight. For this purpose, polyacrylamide gels with different content of acrylamide, respectively with different pore size were used. A system with two phases (stacking and resolving gels) was employed:

Stacking gel 5 % acrylamide (mixture for 4 mini-gels)

Reagent	Volume (mL)
Millipore water	5.50
Rotiphorese Gel 30 (37.5:1)	1.30
TRIS 1.5 M, pH= 6.8	1.00
APS solution (10 %)	0.08
SDS solution (10 %)	0.08
TEMED	0.006

Resolving gel 10 % acrylamide (mixture for 4 mini-gels)

Reagent	Volume (mL)
Millipore water	7.90
Rotiphorese Gel 30 (37.5:1)	6.70
TRIS 1.5 M, pH= 8.8	5.00
APS solution (10 %)	0.20
SDS solution (10 %)	0.20
TEMED	0.008

Resolving gel 8 % acrylamide (mixture for 4 mini-gels)

Reagent	Volume (mL)
Millipore water	9.26
Rotiphorese Gel 30 (37.5:1)	5.34
TRIS 1.5 M, pH= 8.8	5.00
APS solution (10 %)	0.20
SDS solution (10 %)	0.20
TEMED	0.02

Different pore-sized resolving gels were used in order to analyze proteins with different molecular weight. Resolving gels with higher acrylamide content (10 %) and respectively smaller pore-size were typically used for separation and by subsequent analysis of proteins under 100 kDa (i.e. Akt, AMPK, and β-Actin). Bigger pore-sized resolving gels (8 %) were used by analysis of proteins with molecular weight above 100 kDa (i.e eNOS). Stacking (5 % acrylamide) gels were always used to pre-concentrate the sample proteins before resolving, which resulted in sharper bands by Western blot detection.

The resolving gel was always casted first. Before its polymerization occurred, it was overlaid with Millipore water, which resulted in a smooth and regular interfacial

separation. Upon removal of the water, the stacking gel was casted over the resolving one. After complete polymerization, the formed upper gel pockets were rinsed multiple times with 1 x running buffer in order to eliminate any residual acrylamide, which had not been polymerized.

Typically up to nine pockets were loaded with samples with equal protein concentrations (20-30 µg/lane) and one pocket was loaded with pre-stained protein standard (C-2.1.3.1).

For the electrophoresis performance the gel-electrophoresis equipment (described under C-2.2.2) and 1 x running buffer (C-2.3.1) were used. A direct current power supply provided constantly 180 V and the process continued until the protein standards were well separated from each other (typically 1 – 1.5 h).

2.4.4.2 Total protein staining of gels

If necessary, polyacrylamide gels underwent total protein staining after separation for normalization as a loading control. Its superiority in comparison to housekeeping proteins (i.e. β-actin) has previously been suggested [431]. Briefly, the gel was washed with demineralized water, placed in Coomassie Blue staining solution (indicated under C-2.3.4) and incubated on the shaker for 1 hour. In order to remove the background and make protein bands clear, the gel was washed with destaining solution (C-2.3.4), which was renewed several times (for minimally 2 hours). Stained gels were digitalized employing a high definition camera and were used for evaluation of protein content per lane which served for data normalization. These gels were not further used for protein transfer.

2.4.4.3 Protein transfer (blotting)

After separation of the proteins, gels (C-2.4.4.1) were equilibrated in transfer buffer (C-2.3.1). Wet/tank blotting indicated superior protein transfer, in comparison to semi-dry technique during the method development, and was therefore used for all further experiments. It was performed with the Mini-Trans Blot® cell (C-2.2.2). Pre-soaked gels and nitrocellulose membranes (C-2.1.3.1) were placed between foam pads and blotting papers, air bubbles were eliminated by abundance of buffer and careful pressing the gel against the membrane manually. This “blotting sandwich” was placed into the tank with transfer buffer and cooling pad. The membrane was orientated to the positive pole and the gel to the negative one, as proteins were negatively charged. According to the molecular weight of the protein of interest, the process lasted 1 hour for proteins under 100 kDa and 1 hour and 30 minutes for proteins over 100 kDa and was always performed at 4 °C. The voltage used was 100 V and the current had been limited to 375 mA.

As the blotting step was crucial for the analysis, the protein transfer onto the nitrocellulose membrane was visualized and checked by reversible staining with Ponceau S.

2.4.4.4 Immunodetection

The membrane was blocked, in order to prevent the unspecific binding of antibodies to the nitrocellulose. The blocking agent differed according to the detected proteins. For phospho-Akt (Thr308) and Phospho-Akt (Ser473) 5 % BSA in TBST was used. For all other proteins membranes were blocked with 5 % low-fat dry milk in TBST (C-2.3.1). In both cases the process lasted 1 hour at room temperature. Thereafter, membranes were briefly washed in TBST. Primary rabbit antibodies (C-2.1.3.2) were applied to detect specific epitopes of the proteins of interest. Antibodies were diluted in 5 % BSA-TBST as following:

Antibody	Dilution
Phospho-Akt (Ser473) Antibody	1:1000
Akt Antibody	1:2000
Phospho-Akt (Thr308) 224F9 mAb	1:800
β-Actin (D6A8) mAb	1:10000
Phospho-AMPKα (Thr172) (40H9)	1:2000

The incubation was typically performed at 4 °C overnight on the shaker, or alternatively for 2 hours at room temperature.

To remove the unbound primary antibodies, membranes underwent four washing steps with TBST on a shaker, 10 minutes each. A secondary anti-rabbit antibody, linked with HRP recognized the primary ones and was applied at a dilution of 1:10000 for 2 hours at room temperature (or at 4 °C overnight, alternatively). In order to reduce signal/noise ratio membranes underwent further four washing steps with TBST, each lasting 10 minutes.

Due to recognition of the membrane-bound antigen by primary antibody, which in turn was recognized by the HRP-marked secondary antibody, a chemiluminescent detection was possible. Each membrane was developed by incubation with equal parts of stabilized peroxide solution and luminol/enhancer solution of the ECL (enhanced chemiluminescent) substrate (C-2.1.3.3). In the linear dynamic range the signal was proportional to the amount of antigen in probes and was detected by FluorChem FC2 Doku imaging system (C-2.2.2).

The obtained images were quantified densitometrically by means of ImageJ [432].

2.4.4.5 Reprobing (stripping) of Western blot

Reprobing of Western blot is a technique which enables multiple use of the same membrane for detection of target proteins. After detection of a phosphorylated form of a given protein, reprobing was used to detect total (pan) form of this protein, regardless of its phosphorylation status. By means of normalization of the phosphorylation level to the pan protein level from the same membrane, the influence of differences from protein

concentrations, uneven gel loading or transfer was minimized, which lead to better reproducibility of data.

After development of the membrane, it was incubated in stripping buffer (20 minutes, room temperature) (C-2.3.3) in order to remove primary and secondary antibodies from it. Afterwards, the membrane underwent four washing steps with TBST, each for 10 minutes, to ensure complete removal of the stripping buffer. Then, unspecific binding sites of the membrane were blocked with milk-containing buffer for 2 hours at room temperature. Further immunodetection with appropriate antibodies was performed as described above (C-2.4.4.4).

2.4.5 Quantitative analysis of Akt-phosphorylation status

For a screening of the effects of polyphenols on Akt-signaling, quantitative analysis of the phosphorylation status of this kinase in cell lysates was carried out employing a commercially available "sandwich"-ELISA (C-2.1.4). The previously described standard protocol (C-2.4.1.4) was slightly modified: cells were not incubated in a serum-free medium (starved) before treatment with polyphenols. In this way it was investigated whether the substances of interest were capable to antagonize the elevated Akt-phosphorylation by the growth factors contained in the standard medium supplemented with 10 % fetal bovine serum from the same batch 1107A. Due to the chemical instability of polyphenols [272] their effects were determined for short incubation times (5 minutes) at a concentration of 10 µM. Cells were lysed using 300 µL (1x) lysis buffer/well. The latter was provided within the ELISA-kit and was supplemented with protease and phosphatase inhibitors (listed under C-2.1.2.2).

The immunoassay was performed according to the manufacturer's protocol with some deviations. Lysates were diluted 1:3 with (1x) assay diluent and were added to assay wells and incubated overnight at 4 °C. In each experiment the lysate from DMSO-treated cells (corresponding to the 100 % control) and its further 1:1 dilution (50 % control) were used as reference standards. Detection antibody against phospho-Akt (Ser473) was diluted 1:55 in (1x) assay diluent as suggested by manufacturer, while pan Akt antibody was diluted 1:220 in order to avoid high readouts outside the linear range of the assay. After obtaining of optical densities and subtraction of blanks, the values (in %) for phospho-Akt (Ser473) and pan Akt were calculated using the reference standards. Then data for phospho-Akt (Ser473) were normalized to those of pan Akt for the respective lysates.

2.4.6 Mycoplasma test

Mycoplasma tests were regularly performed in order to verify that the cells were free of contamination. Therefore, the LookOut® Mycoplasma PCR Detection Kit (C-2.1.6) was utilized. This assay was based on polymerase chain reaction (PCR) and possessed high sensitivity regarding detection of bright spectrum of mycoplasma species, which had been

described as common cell culture contaminants. Samples were acquired from supernatants of confluent cell cultures. The kit's primer set was specific for a defined highly conserved coding region in the mycoplasma genome. Samples underwent a PCR according to the producer's protocol. Products of the reaction along with negative and positive controls supplied in the kit were pipetted into a 1 % agarose gel with 0.02 % DAPI. Electrophoresis was conducted for 1 hour at 140 V in 1 x TBE buffer (C-2.3.6). The amplified DNA fragments were visualized with DAPI-stain under UV-light (Figure 46).

Figure 46: Representative image of polymerase chain reaction (PCR)-based mycoplasma test. Samples derived from cell culture supernatants underwent amplification and agarose gel electrophoresis. The first lane was a negative control for mycoplasma species and the second one was a positive control. Accordingly, both of the samples were negative.

2.5 *In silico* modeling

For the *in silico* modeling described under B-2.5 the Molecular Operating Environment program (MOE), version 2018.01 was used [96]. The functionality "Flexible Alignments" is based on a feature probability density of input molecules' conformations and the generation of overlays takes place in three stages: perturbation, optimization and comparison [312]. A scoring was used to evaluate the quality of the created superpositions, among which the most important measures were average strain energy (U), similarity of shape (F), and ground alignment score (S; dependent on U and F). The lower is the value of S the better is the alignment's quality [313]. For the similarity following features of input molecules were considered: acid/base properties, partial charges, H-bound donors/acceptors, hydrophobicity, molecular volumes, etc. Default settings were used. Certain limits were set: Attempts to create new alignment ≤ 20 times, the potential energy of alignment to < 7 kcal/mol, number of alignments in collection < 100.

All databases used for virtual screening were prepared by Mr. Mathias Diebold, MSc (research group of Prof. Dr. Sotriffer). Initially, databases were *lead-like*- (criteria similar to Lipinski's 5 rule) filtered. Important properties to consider were: number of N and O atoms that are H-bound donors (must be five or less); cut-off for molecular weight: ≤ 450 – accepted; good solubility (logP); the number of rotatable bounds (must be 10 or less); number of rings. Other filters applied were *non-reactive* (sorting out molecules with

reactive groups such as aldehydes, thiols, epoxides, etc.) and *smallring* (excluding compounds with rings of size nine or more atoms). Afterwards, 3D-conformers of the molecules were generated using default settings and limiting the potential energy to 10 kcal/mol. To establish if the pharmacophore-model-matching conformations are energetically favorable (B-2.5.6), a 3D systematic conformational search was performed and the relative conformer energy was set to be less than 7 kcal/mol.

The solubility (LogP, $LogD_{7.4}$), acid-base character (pK_a and pK_b-values), ionization states, and tautomerization, relevant for the post-screening analysis (B-2.5.6) were predicted by MoKa software, version 3.1.3 [99].

3 Effects of polyphenols on DPP IV activity and expression

3.1 Effects of polyphenols on the enzymatic activity of DPP IV

3.1.1 Chemicals and reagents

DPP IV-Inhibitor-Screening-Kit	Cayman Chemicals, USA

3.1.2 Materials and devices

All fluorescent measurements were performed with the same equipment as described in C-1.3.1.

3.1.3 Experimental design

3.1.3.1 Classic protocol

The potential of polyphenols to inhibit the enzymatic activity of DPP IV was analyzed using the DPP IV-Inhibitor-Screening-Kit. The method has been developed by Fujiwara and Tsuru (1978) [418], which has already been described (see C-1.4.5). The fluorescent signal of the proteolytic reaction product (AMC) was directly proportional to the enzyme activity. The initial enzyme activity (100 %) was assumed to be the activity of defined amount purified human recombinant DPP IV without presence of any inhibitor. The activity after addition of a given polyphenolic compound or referent DPP IV-inhibitor at a defined concentration was compared to the initial enzyme activity (100 %) and thus the inhibitor activity of the compound of interest was defined, using the following formula:

$$\% \text{ Inhibition} = [(F_{100\,\%} - F_{\text{Inhibitor}})/F_{100\,\%}] \text{ x } 100, \text{ where}$$

$F_{100\,\%}$ represents the fluorescence measured by the enzyme without any inhibitors added, while $F_{\text{Inhibitor}}$ was the fluorescence measured in presence of any investigated inhibitor. 50 nM sitagliptin (final concentration) was used as a positive control. Each experiment was performed in triplicate.

3.1.3.2 Modified protocol

In order to improve the clinical relevance of the investigation of DPP IV in human serum, a modified approach which better reflected physiological conditions was developed. For this purpose, the endogenous DPP IV activity of human serum (pooled from three donors,

n= 3) was used to perform the screening. The serum was diluted 10 times with assay buffer, in order to reduce its matrix effect. This assay was validated using the reference substance sitagliptin in the nanomolar concentration range (Figure 47).

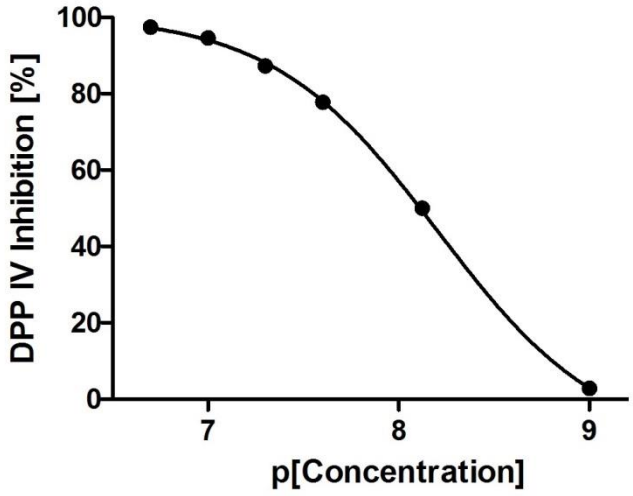


Figure 47: Concentration-Response curve of the inhibitory effect of sitagiliptin on the serum DPP IV activity in the modified approach of DPP IV inhibitor assay (n= 2). The data was analyzed with Graph Pad Prism v 6.0. The curve was fitted using non-linear regression.

3.2 Effects of polyphenols on the DPP IV-Expression

3.2.1 Cells, chemicals and reagents

3T3-L1 is a murine embryonal fibroblast cell line which has pre-adipocyte properties and can differentiate into adipocytes under defined conditions. These cells are named after known cultivation protocol "3-day transfer, inoculum 3×10^5 cells" and were derived through a clonal isolation [433, 434]. Mature fat cells are morphologically different than pre-adipocytes and during the maturation the number and the size of lipid droplets is increasing. An aliquot of these cells with passage 21 was purchased from Sigma-Aldrich and obtained from the ECACC collection of Public Health England.

3.2.1.1 Specific reagents for cell culture and media

FBS i. (fetal bovine serum – heat-inactivated)	Gibco/Thermo Fisher Scientific, USA
FBS n.i. (non-inactivated)	Sigma-Aldrich, USA
Insulin solution human (10 mg/mL)	
Dexamethasone	
IBMX (3-isobutyl-1-methylxanthine)	Fluka BioChemika, Switzerland
Rosiglitazone	Alexis Biochemicals, Switzerland

Stock solutions of IBMX (250 mM), dexamethasone (1 mM), and rosiglitazone (4.5 mM) in DMSO were used.

Following media were freshly prepared according to the protocol created by Zebisch et al. [435] with minor modifications and used for cultivation and differentiation of 3T3-L1 cells:

Medium	Content
Cultivation medium (CM)	HG DMEM (high glucose) 8 % (v/v) FBS i. 2 mM L-glutamine 1 mM sodium pyruvate 1 % Pen/Strep
Differentiation medium (DMR)	HG DMEM 10 % (v/v) FBS n. i. 0.5 mM IBMX 1 µM dexamethasone 2 µM Rosiglitazone 20 µg/mL insulin 2 mM L-glutamine 1 mM sodium pyruvate 1 % Pen/Strep
Insulin medium (IM)	HG DMEM 10 % (v/v) FBS n. i. 20 µg/mL insulin 2 mM L-glutamine 1 mM sodium pyruvate 1 % Pen/Strep
Post-differentiation medium (PDM)	HG DMEM 10 % (v/v) FBS n. i. 2 mM L-glutamine 1 mM sodium pyruvate 1 % Pen/Strep

For the origin of additional reagents used in cell culture media formulation please refer to C-2.1.2.2

3.2.1.2 Specific reagents for Oil Red O staining

Oil Red O	Sigma-Aldrich, USA
Formalin solution, neutral buffered, 10 % (v/v)	
PBS	
Isopropanol	Inventory at the Faculty of Chemistry and Pharmacy (University of Würzburg)

3.2.1.3 Specific reagents for ELISA analysis

Mouse DPPIV/CD26 DuoSet ELISA	R&D Systems, Inc., USA
DuoSet ELISA Ancillary Reagent Kit	

3.2.2 Materials and devices

3.2.2.1 Materials and devices for cell culture

Slide-a-lyzer™, cut-off: 10 kDa, 0.5 – 2 mL	Thermo Fisher Scientific, Waltham, MA, USA
Freeze dryer Alpha 1-2 LDplus	Analis, Belgium

Further materials and devices used for cell culture model were described under C-2.2.1.

3.2.2.2 Materials and devices for Oil Red O staining

Round paper filter MN615	Macherey-Nagel GmbH, Germany
0.2 µm cellulose acetate membrane filter	VWR International, NY, USA
High definition camera (8 MP)	Lenovo Group Ltd., Beijing, China

3.2.2.3 Materials and devices for ELISA

The equipment used for ELISA measurements is described under C-2.2.3.

3.2.3 Experimental set-up: cell culture model

A potential influence of polyphenolic compounds on the expression of DPP IV was investigated *in vitro* in a cell culture model. For this purpose 3T3-L1 cells were differentiated into mature adipocytes. Cells were grown and induced using an established protocol [435] with some modifications, aiming maximal conversion rate. Briefly, cells were thawed as previously described (C-2.4.1.2) and cultivated in medium containing 8 % heat-inactivated FBS (CM; see C-3.2.1.1). Once they reached 70 % confluence they were splitted at a ratio of 1:8. This procedure was repeated every third day to keep the cells in the proliferative state. This was essential as reaching confluence during cultivation caused growth inhibition and influenced differentiation negatively. For differentiation cells were seeded on gelatin-coated 6-well plates and grown to confluence. After two days they were treated with induction medium containing adipogenic cocktail of IBMX, insulin and dexamethasone (DMR; see C-3.2.1.1). Since the purchased cells were from a high passage (21) additionally the differentiation-enhancing compound rosiglitazone (PPARγ-agonist and insulin sensitizer) was added at a concentration of 2 µM. Deviating from the original protocol cells were incubated in DMR not for 48, but for 96 hours to achieve a good differentiation rate. The DMSO concentration was kept as low as possible (0.35 % v/v).

After this period the medium became viscous due to the lipid secretion by differentiating pre-adipocytes. The medium was removed carefully in order to keep an intact cellular monolayer and was replaced with medium containing 10 % non-inactivated serum and 20 mg/mL insulin (IM; see C-3.2.1.1) for 96 h. Thereafter, medium was exchanged with post-differentiation medium (PDM; see C-3.2.1.1) containing 10 % non-inactivated FBS. Cells were maintained in this medium for 4-6 days until a 70-90 % differentiation rate was achieved. During this period medium was exchanged every second day. Cellular differentiation and lipid accumulation was verified trough Oil Red O staining (C-3.2.4). The differentiation process is summarized in Figure 48.

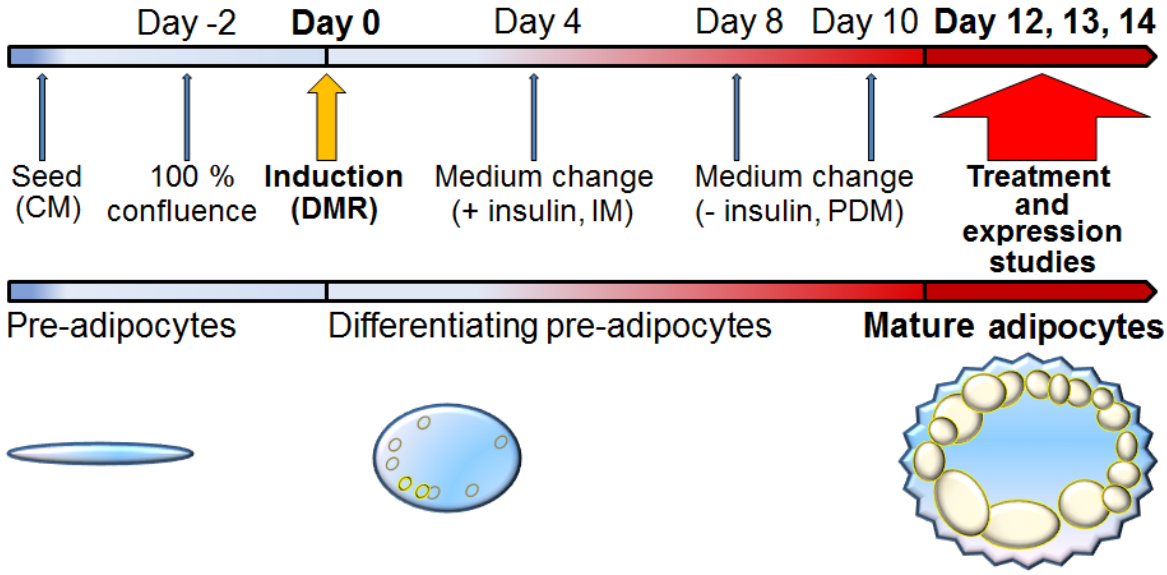


Figure 48: Differentiation timetable of 3T3-L1. Cells were seeded in cultivation medium (CM). Two days upon confluence differentiation was induced in adipogenic medium (DMR). After four days cells were maintained in medium containing insulin (IM) and started to change their morphology and to accumulate lipids. After additional four days cells were kept in growth medium (PDM) which was changed every second day. 12-14 days after induction cells were treated with investigated substances or vehicle for 72 h and were subjected to DPP IV-expression analysis.

Some factors used for optimization of the cell conversion process which were experimentally established or had already been described in the literature (Table 14). Plates used for differentiation were coated with 1% gelatin solution (C-2.3.5) in order to minimize the effect of the cell culture dish surface. The tested non-inactivated FBS showed slight superiority as a proadipogenic nutrient compared to the inactivated one.

Table 14.: Beneficial factors for pre-adipocyte differentiation:

Factor	Importance	Evidence
Possibly lower passage	Essential	[435]
Two days post-confluence	Essential	[436]
Presence of rosiglitazone	Important for high cell passages	[435]
Additional 48 h incubation in DMR and IM	Essential	Experimentally established
Elevated insulin concentration (20 µg/mL)	Important	Experimentally established
Coating of the cell culture dish surface	Important	[437]
Smaller medium volume	Secondary	[438]
Lowest DMSO concentration possible	Secondary	Experimentally established

After phenotype conversion (usually 12-14 days after induction) cells were treated with compounds of interest or vehicle. Polyphenols were tested at a concentration of 10 µM. Medium containing compounds was changed every 24 h up to 72 h (3 days) of treatment. On the 48th hour medium was replaced with 1 mL 1% BSA medium to investigate whether the tested substances were able to influence DPP IV release from adipocytes in cell culture medium.

To investigate the expression of DPP IV on the protein level cells were lysed as described under C-2.4.2 using 300 µL/well RIPA buffer supplemented with PI (protease inhibitors). Cell lysates' supernatants obtained after centrifugation (4 °C, 10 minutes, 18000 g) underwent ELISA analysis (see C-3.2.5).

Conditioned (24 h) medium was collected before lysis and was investigated for DPP IV liberation by adipocytes. Due to very low DPP IV concentrations in conditioned medium it was firstly dialyzed by a Slide-a-lyzer™ with PBS overnight and then freeze-dried and reconstituted in 150 µL Millipore water. Thus medium was finally concentrated 6.7 times. 100 µL of the reconstitute were used for ELISA (C-3.2.5).

3.2.4 Oil Red O staining protocol

Cellular lipids were stained with Oil Red O using previously described protocol [439]. A stock solution of 0.3 % (w/v) Oil Red O in 100 % isopropanol was prepared. Prior to use this solution it was diluted with Millipore water at a ratio of 6:4, resulting in a 0.18 % (w/v) Oil Red O working solution. After 10 minutes the latter was filtered through a round paper filter and then through a 0.2 µm pore-sized cellulose acetate filter. Meanwhile cells were fixed with a 10 % (v/v) formalin solution for 30 minutes and washed thrice with PBS and once with 60 % (v/v) isopropanol, and subsequently air-dried. Afterwards the Oil Red O working solution was applied for 15 minutes. After removal the cells were washed 4 times with PBS to remove any rests of the stain. Cells were examined under a light microscope (C-2.2.1). All steps were carried out at room temperature. Pictures were taken with a high definition camera listed under C-3.2.2.2 (Figure 49).

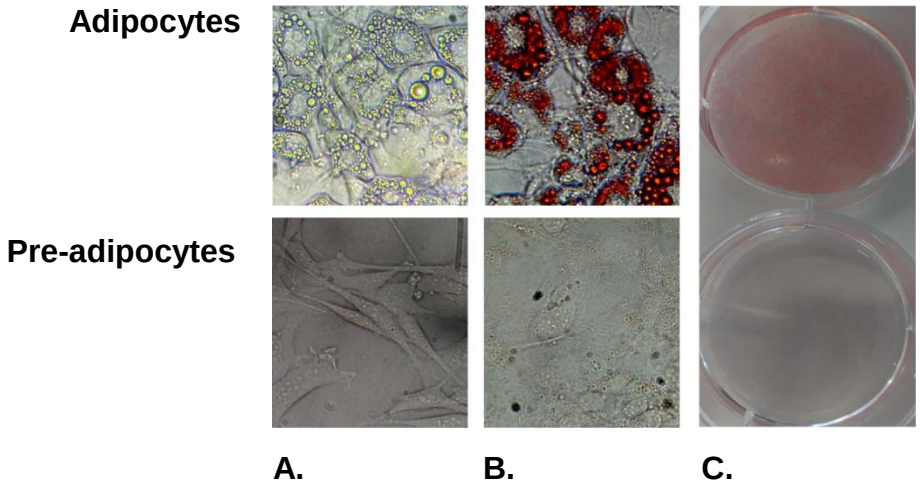


Figure 49: Differences between 3T3-L1 cells before (pre-adipocytes) and after (adipocytes) differentiation. A. Cell morphology. Lipid accumulation staining by Oil Red O: B. Microscopically. C. Macroscopically.

3.2.5 Experimental set-up: ELISA analysis

DPP IV concentrations in cell culture samples were determined employing an indirect sandwich Mouse DPPIV/CD26 DuoSet ELISA according to the manufacturers' protocol with some deviations. Briefly, strip wells were coated overnight at room temperature (RT) with 4 µg/mL capture antibody diluted in PBS. After washing wells were blocked with 1 % BSA in PBS for at least 1 h (RT). Lysates were diluted 1:50 to 1:100 with (1x) the supplied dilution reagent. Reconstitutes from conditioned medium were analyzed undiluted. Samples were added to the wells along with standards and incubated for 2 hours (RT). A biotinylated anti-mouse DPP IV antibody was used for detection of immobilized antigens. Deviating from the original protocol, streptavidin-HRP (Str-HRP) conjugate was diluted 1:400 instead of 1:200, as the suggested concentration resulted in higher blank values. After removal of the unbound Str-HRP, chromogen substrate was added to each well,

reaction was stopped and optical density was measured at the wavelength of 450 nm. Concentrations of samples were calculated by extrapolation from standard curve of mouse DPP IV protein referent standards provided by the producer. Standard curves were obtained within each experimental run. A representative standard curve for Mouse DPPIV/CD26 DuoSet ELISA is shown in Figure 50.

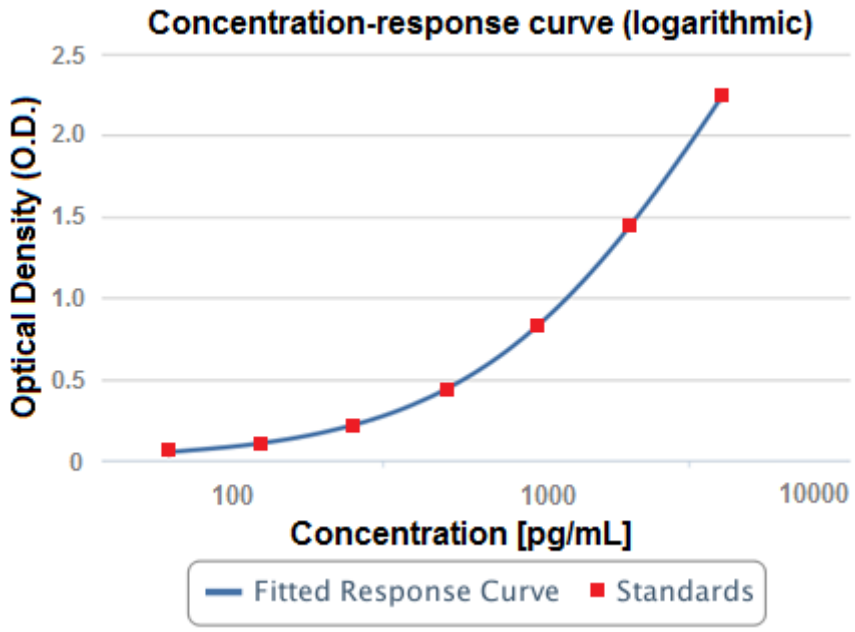


Figure 50: Representative standard curve for the Mouse DPPIV/CD26 DuoSet ELISA. The data was analyzed with free online ELISA Software [415]. The curve was fitted using 5-parameter logistic regression. On the x-axis the logarithmic values of mouse DPP IV concentrations (in pg/mL) were plotted and on the y-axis the optical density values (O.D.) after subtraction of the experimental blank. The absorbance was measured at the wavelength of 450 nm. The average accuracy of the standard curve was 100.04 %. The unknown sample concentrations were calculated from their optical density values in regard with the standard curve.

4 Interactions of polyphenols with fluorescence

4.1 Chemicals and reagents

Assay Buffer	NaCl	100 mM
	EDTA	1 mM
	Tris-HCl	20 mM
		pH= 8.0
Human recombinant DPP IV	Obtained from DPP IV-Inhibitor-Screening-Kit, Cayman Chemicals, USA, C-3.1.1	
Human serum	See C-3.1.3.2	
6-methoxyflavone Quercetin Urolithin A Urolithin B	For the origin of compounds refer to C-2.1.2.3	

4.2 Materials and devices

All fluorescent measurements were performed with the same equipment as described in C-1.3.1. Heracell, 150I, cell culture incubator and horizontal mini shaker (listed under C-1.3) were used as well.

4.3 Fluorescence interactions analysis

Possible interactions of given compounds with fluorescence were analyzed under relevant conditions close to those of experimental approaches investigating DPP IV inhibition potential employing DPP IV-Inhibitor-Screening-Kit (C-3.1.3.1) and the modified method (C-3.1.3.2). Fluorescent measurements were conducted at $\lambda_{excitation/emission}$ = 360 nm/460 nm and were registered before (0 min) and after (30 min) incubation at 37 °C (shaking) and in the presence and absence of the fluorescent compound AMC. Fluorescent properties of the given compounds were investigated respectively in three different environments: assay buffer, DPP IV dissolved in assay buffer (referred as DPP IV) and human serum diluted in assay buffer (1:10). As a negative control a vehicle (DMSO) was used at a concentration of 0.5 % (v/v).

D Supplement

1 Section 1 – Clinical study

1.1 GLP-1 (active) serum concentrations

1.1.1 GLP-1 (active) – before outlier exclusion

GLP-1 (active) concentrations [pM]								
Volunteer	T1-0	T1-30	T3-0	T3-30	T2-0	T2-30	T4-0	T4-30
1	7.80	7.09	9.46	9.59	11.54	7.76	9.95	6.87
2	9.66	11.29	8.91	11.38	16.69	17.06	12.24	7.56
3	10.41	7.66	10.16	9.84	3.15	6.66	4.52	2.51
4	7.52	12.29	7.57	14.17	8.21	9.92	2.80	6.25
5	10.32	9.53	5.00	4.50	6.82	4.79	4.60	0.86
6	12.81	14.02	10.38	12.21	9.69	8.16	2.83	2.15
7	9.55	7.17	14.05	13.57	3.52	3.63	4.93	6.05
8	31.76	23.66	28.13	27.01	24.92	24.01	23.45	19.18
9	12.32	9.67	7.75	11.52	9.57	8.25	10.41	11.18
10	10.33	9.79	10.14	9.05	3.28	5.58	8.43	13.25
11	3.48	9.28	11.14	5.58	6.33	10.57	7.26	10.04
12	3.53	8.14	5.11	5.24	3.46	5.44	6.21	10.34
13	7.39	8.62	12.11	7.06	10.40	9.47	8.64	7.28
14	7.74	12.82	0.82	13.03	6.53	11.13	13.59	12.86
15	10.16	11.95	11.03	12.01	12.88	16.06	10.48	11.59
16	10.82	11.92	10.03	12.59	10.36	11.54	11.01	14.85
17	9.18	13.64	9.22	14.05	10.89	16.09	10.88	16.38
18	7.14	7.85	11.99	12.25	12.22	15.84	7.06	6.69
19	4.82	3.34	4.68	5.08	4.69	9.06	7.19	6.56
20	14.76	13.56	5.76	6.25	18.35	16.62	14.18	12.29

Descriptive statistical parameters of GLP-1 (active) classic and rice OGTT protocol data (n for each experimental state shown in the table), outliers detection and exclusion

Phase	Mean (pM)	S.D.	Median	Mean Dev.	S.E.M.	n
T1-0	10.07	5.87	9.61	3.30	1.31	20
T1-30	10.66	4.10	9.73	2.92	0.92	20
T3-0	9.67	5.36	9.75	3.24	1.20	20
T3-30	10.80	5.01	11.45	3.50	1.12	20
T2-0	9.67	5.60	9.63	4.12	1.25	20
T2-30	10.88	5.22	9.70	4.13	1.17	20
T4-0	9.03	4.76	8.54	3.49	1.06	20
T4-30	9.24	4.82	8.80	3.96	1.08	20

Criteria: Outlier > 2.5 S.D., Grubbs' Test
Outliers: 8-T1-0, 8-T1-30, 8-T2-0, 8-T2-30, 8-T3-0, 8-T3-30, 8-T4-0
Statistical significance: n.s.

1.1.2 Serum concentrations of GLP-1 (active) - outliers excluded

GLP-1 (active) concentrations [pM]								
Volunteer	T1-0	T1-30	T3-0	T3-30	T2-0	T2-30	T4-0	T4-30
1	7.80	7.09	9.46	9.59	11.54	7.76	9.95	6.87
2	9.66	11.29	8.91	11.38	16.69	17.06	12.24	7.56
3	10.41	7.66	10.16	9.84	3.15	6.66	4.52	2.51
4	7.52	12.29	7.57	14.17	8.21	9.92	2.80	6.25
5	10.32	9.53	5.00	4.50	6.82	4.79	4.60	0.86
6	12.81	14.02	10.38	12.21	9.69	8.16	2.83	2.15
7	9.55	7.17	14.05	13.57	3.52	3.63	4.93	6.05
8	Outlier	Outlier	Outlier	Outlier	Outlier	Outlier	Outlier	19.18
9	12.32	9.67	7.75	11.52	9.57	8.25	10.41	11.18
10	10.33	9.79	10.14	9.05	3.28	5.58	8.43	13.25
11	3.48	9.28	11.14	5.58	6.33	10.57	7.26	10.04
12	3.53	8.14	5.11	5.24	3.46	5.44	6.21	10.34
13	7.39	8.62	12.11	7.06	10.40	9.47	8.64	7.28
14	7.74	12.82	0.82	13.03	6.53	11.13	13.59	12.86
15	10.16	11.95	11.03	12.01	12.88	16.06	10.48	11.59
16	10.82	11.92	10.03	12.59	10.36	11.54	11.01	14.85
17	9.18	13.64	9.22	14.05	10.89	16.09	10.88	16.38
18	7.14	7.85	11.99	12.25	12.22	15.84	7.06	6.69
19	4.82	3.34	4.68	5.08	4.69	9.06	7.19	6.56
20	14.76	13.56	5.76	6.25	18.35	16.62	14.18	12.29

Descriptive statistical parameters of GLP-1 (active) classic and rice OGTT protocol data (n for each experimental state shown in the table) - outliers excluded.

Phase	Mean (pM)	S.D.	Median	Mean Dev.	S.E.M.	n
T1-0	8.93	2.97	9.55	2.32	0.68	19
T1-30	9.98	2.8	9.67	2.28	0.64	19
T3-0	8.7	3.23	9.46	2.55	0.74	19
T3-30	9.95	3.34	11.38	2.88	0.77	19
T2-0	8.87	4.42	9.57	3.56	1.01	19
T2-30	10.19	4.33	9.47	3.51	0.99	19
T4-0	8.27	3.43	8.43	2.85	0.79	19
T4-30	9.24	4.82	8.8	3.96	1.08	20

Statistical significance: n.s.

1.1.3 Individual change in GLP-1 (active) between T1-T3 and T2-T4

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T1/1-T3	-21.33	1-T2-0/ 1-T4-0	13.78	11-T1-0/ 11-T3-0	Outlier %	11-T2-0/ 11-T4-0	-14.69
2-T1/2-T3	7.76	2-T2-0/ 2-T4-0	26.66	12-T1-0/ 12-T3-0	-44.76	12-T2-0/ 12-T4-0	-79.48
3-T1/3-T3	2.40	3-T2-0/ 3-T4-0	-43.50	13-T1-0/ 13-T3-0	-63.87	13-T2-0/ 13-T4-0	16.92
4-T1/4-T3	-0.66	4-T2-0/ 4-T4-0	65.86	14-T1-0/ 14-T3-0	89.39	14-T2-0/ 14-T4-0	Outlier %
5-T1/5-T3	51.54	5-T2-0/ 5-T4-0	32.52	15-T1-0/ 15-T3-0	-8.56	15-T2-0/ 15-T4-0	18.63
6-T1/6-T3	18.97	6-T2-0/ 6-T4-0	70.75	16-T1-0/ 16-T3-0	7.30	16-T2-0/ 16-T4-0	-6.27
7-T1/7-T3	-47.14	7-T2-0/ 7-T4-0	-40.31	17-T1-0/ 17-T3-0	-0.44	17-T2-0/ 17-T4-0	0.09
8-T1/8-T3	Outlier	8-T2-0/ 8-T4-0	Outlier	18-T1-0/ 18-T3-0	-67.93	18-T2-0/ 18-T4-0	42.23
9-T1/9-T3	37.09	9-T2-0/ 9-T4-0	-8.78	19-T1-0/ 19-T3-0	2.90	19-T2-0/ 19-T4-0	-53.30
10-T1/10-T3	1.84	10-T2-0/ 10-T4-0	Outlier %	20-T1-0/ 20-T3-0	60.98	20-T2-0/ 20-T4-0	22.72

8-T1-0/T3-0, 8-T2-0/T-4-0 - outlied as previously described
10-T2-0/T4-0, 11-T1-0/T3-0, 14-T2-0/T4-0 - outlied ratios

	Control state	Treated state	
Mean (%)	100.00	97.45	n= 35, Mean Individual Change between T1-T3 and T2-T4, time point 0 min: decrease of 2.55 %; *Statistical significance: n.s.*
S.D. (%)	38.66	40.83	

1.2 DPP IV

1.2.1 DPP IV serum concentrations

1.2.1.1 DPP IV serum concentrations before outlier exclusion

Control State, T1	DPP IV conc.	Control State, T2	DPP IV conc.	Treated State, T3	DPP IV conc.	Treated State, T4	DPP IV conc.
Sample	[ng/mL]	Sample	[ng/mL]	Sample	[ng/mL]	Sample	[ng/mL]
1-T1-0	369.08	1-T2-0	726.19	1-T3-0	432.76	1-T4-0	493.83
2-T1-0	535.77	2-T2-0	426.38	2-T3-0	397.99	2-T4-0	390.21
3-T1-0	420.07	3-T2-0	421.87	3-T3-0	281.61	3-T4-0	302.13
4-T1-0	451.29	4-T2-0	751.41	4-T3-0	404.1	4-T4-0	480.995
5-T1-0	891.12	5-T2-0	892.70	5-T3-0	773.99	5-T4-0	587.89
6-T1-0	810.47	6-T2-0	635.65	6-T3-0	691.26	6-T4-0	589.93
7-T1-0	1024.10	7-T2-0	669.36	7-T3-0	877.36	7-T4-0	620.27
8-T1-0	760.23	8-T2-0	604.90	8-T3-0	687.3	8-T4-0	722.35
9-T1-0	761.06	9-T2-0	720.91	9-T3-0	547.57	9-T4-0	694.02
10-T1-0	610.99	10-T2-0	546.38	10-T3-0	613.495	10-T4-0	588.7
11-T1-0	561.96	11-T2-0	510.95	11-T3-0	543.99	11-T4-0	545.78
12-T1-0	871.71	12-T2-0	601.85	12-T3-0	774.297	12-T4-0	517.11
13-T1-0	1022.92	13-T2-0	1545.67	13-T3-0	1174.96	13-T4-0	1511.26
14-T1-0	991.04	14-T2-0	862.20	14-T3-0	1143.26	14-T4-0	592.76
15-T1-0	980.98	15-T2-0	1066.09	15-T3-0	1063.9	15-T4-0	1583.66
16-T1-0	735.99	16-T2-0	821.16	16-T3-0	1066.09	16-T4-0	1264.25
17-T1-0	1128.67	17-T2-0	1449.74	17-T3-0	813.83	17-T4-0	1029.32
18-T1-0	522.73	18-T2-0	786.74	18-T3-0	518.51	18-T4-0	779.62
19-T1-0	1261.77	19-T2-0	1177.31	19-T3-0	790.32	19-T4-0	989.27
20-T1-0	876.29	20-T2-0	788.53	20-T3-0	767.24	20-T4-0	702.195

Descriptive statistics of the data, outliers detection and exclusion - DPP IV concentrations

Phase	Mean (ng/mL)	S.D.	Median	Mean Dev.	S.E.M.	n
Control State (T1, T2)	789.86	276.15	760.65	212.32	43.66	40
Treated State (T3,T4)	733.73	305.41	689.28	233.14	48.29	40

Criteria:
Outlier > 2.5 S.D.
Outliers: 13-T2-0, 13-T4-0, 15-T4-0, 17-T2-0

1.2.1.2 Serum concentrations of DPP IV in volunteers - outliers excluded

	Control State (T1, T2)	Treated State (T3,T4)
1-T1-0/1-T3-0	369.08	432.76
2-T1-0/2-T3-0	535.77	397.99
3-T1-0/3-T3-0	420.07	281.61
4-T1-0/4-T3-0	451.29	404.1
5-T1-0/5-T3-0	891.12	773.99
6-T1-0/6-T3-0	810.47	691.26
7-T1-0/7-T3-0	1024.10	877.36
8-T1-0/8-T3-0	760.23	687.3
9-T1-0/9-T3-0	761.06	547.57
10-T1-0/10-T3-0	610.99	613.495
11-T1-0/11-T3-0	561.96	543.99
12-T1-0/12-T3-0	871.71	774.297
13-T1-0/13-T3-0	1022.92	1174.96
14-T1-0/14-T3-0	991.04	1143.26
15-T1-0/15-T3-0	980.98	1063.9
16-T1-0/16-T3-0	735.99	1066.09
17-T1-0/17-T3-0	1128.67	813.83
18-T1-0/18-T3-0	522.73	518.51
19-T1-0/19-T3-0	1261.77	790.32
20-T1-0/20-T3-0	876.29	767.24
1-T2-0/1-T4-0	726.19	493.83
2-T2-0/2-T4-0	426.38	390.21
3-T2-0/3-T4-0	421.87	302.13
4-T2-0/4-T4-0	751.41	480.995
5-T2-0/5-T4-0	892.70	587.89
6-T2-0/6-T4-0	635.65	589.93
7-T2-0/7-T4-0	669.36	620.27
8-T2-0/8-T4-0	604.90	722.35
9-T2-0/9-T4-0	720.91	694.02
10-T2-0/10-T4-0	546.38	588.7
11-T2-0/11-T4-0	510.95	545.78
12-T2-0/12-T4-0	601.85	517.11
13-T1-0/13-T3-0	Outlied	Outlied
14-T2-0/14-T4-0	862.20	592.76
15-T2-0/15-T4-0	see 15-T2/17-T4	Outlied
15-T2-0/17-T4-0	1066.09	1029.32
16-T2-0/16-T4-0	821.16	1264.245
17-T2-0/17-T4-0	Outlied	see 15-T2/17-T4
18-T2-0/18-T4-0	786.74	779.62
19-T2-0/19-T4-0	1177.31	989.27
20-T2-0/20-T4-0	788.53	702.195

Descriptive statistical parameters of the data for DPP IV serum levels.

	Mean (ng/mL)	S.D.	Median	Mean Dev.	n
Control state (T1,T2)	752.60	227.48	755.82	182.93	38
Treated state (T3,T4)	690.91	246.12	653.79	193.75	38

Statistical significance: p= 0.032

1.2.1.3 DPP IV serum concentrations individual changes

For B-1.3.2.1 (Serum concentrations of DPP IV in healthy individuals, individual changes) after exclusion of the outliers the values 15-T2-0 and 17-T4-0 were combined and the ratio between them was calculated.

Individual change (%) in DPP IV concentrations between states T1-T3 and T2-T4:

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T1/1-T3	17.25	1-T2/1-T4	-32	11-T1/11-T3	-3.2	11-T2/11-T4	6.82
2-T1/2-T3	-25.72	2-T2/2-T4	-8.48	12-T1/12-T3	-11.17	12-T2/12-T4	-14.08
3-T1/3-T3	-32.96	3-T2/3-T4	-28.38	13-T1/13-T3	14.86	13-T2/13-T4	Outlier
4-T1/4-T3	-10.46	4-T2/4-T4	-35.99	14-T1/14-T3	15.36	14-T2/14-T4	-31.25
5-T1/5-T3	-13.14	5-T2/5-T4	-34.14	15-T1/15-T3	8.45	15-T2/17-T4	-3.45
6-T1/6-T3	-14.71	6-T2/6-T4	-7.19	16-T1/16-T3	Outlier %	16-T2/16-T4	Outlier %
7-T1/7-T3	-14.33	7-T2/7-T4	-7.33	17-T1/17-T3	-27.89	-	Outlier
8-T1/8-T3	-9.59	8-T2/8-T4	19.42	18-T1/18-T3	-0.81	18-T2/18-T4	-0.91
9-T1/9-T3	-28.05	9-T2/9-T4	-3.73	19-T1/19-T3	-37.36	19-T2/19-T4	-15.97
10-T1/10-T3	0.41	10-T2/10-T4	7.75	20-T1/20-T3	-12.44	20-T2/20-T4	-10.95

13-T2-0, 13-T4-0, 15-T4-0, 17-T2-0 - outlied as previously described
16-T1-0/16-T3-0, 16-T2-0/16-T4-0 - outlied ratios

	Control	Treated	Mean Individual Change between T1-T3 and T2-T4 at time point 0 minute: Decrease of 10.71 % *Statistical significance: p= 0.0003*
Mean (%)	100.00	89.29	
S.D. (%)	31.10	16.06	

1.2.2 DPP IV serum enzymatic activities

1.2.2.1 DPP IV serum enzymatic activities before outlier exclusion

Control State, T1	DPP IV activity	Control State, T2	DPP IV activity	Treated State, T3	DPP IV activity	Treated State, T4	DPP IV activity
Sample	[µU/mL]	Sample	[µU/mL]	Sample	[µU/mL]	Sample	[µU/mL]
1-T1-0	792.42	1-T2-0	1070.72	1-T3-0	664.81	1-T4-0	645.14
2-T1-0	795.38	2-T2-0	1168.63	2-T3-0	815.95	2-T4-0	661.79
3-T1-0	707.23	3-T2-0	650.21	3-T3-0	635.15	3-T4-0	628.59
4-T1-0	787.15	4-T2-0	881.58	4-T3-0	850.99	4-T4-0	665.06
5-T1-0	678.24	5-T2-0	493.32	5-T3-0	547.36	5-T4-0	541.59
6-T1-0	659.52	6-T2-0	658.09	6-T3-0	646.42	6-T4-0	586.96
7-T1-0	680.18	7-T2-0	741.51	7-T3-0	728.49	7-T4-0	572.90
8-T1-0	734.27	8-T2-0	607.82	8-T3-0	641.62	8-T4-0	732.73
9-T1-0	762.25	9-T2-0	701.28	9-T3-0	678.77	9-T4-0	770.40
10-T1-0	752.33	10-T2-0	496.83	10-T3-0	517.39	10-T4-0	498.41
11-T1-0	476.36	11-T2-0	586.30	11-T3-0	660.83	11-T4-0	651.84
12-T1-0	660.83	12-T2-0	769.46	12-T3-0	588.68	12-T4-0	810.87
13-T1-0	652.67	13-T2-0	691.42	13-T3-0	827.79	13-T4-0	1036.11
14-T1-0	609.82	14-T2-0	755.19	14-T3-0	964.62	14-T4-0	528.10
15-T1-0	745.25	15-T2-0	716.26	15-T3-0	937.91	15-T4-0	1108.53
16-T1-0	910.02	16-T2-0	882.79	16-T3-0	816.50	16-T4-0	780.83
17-T1-0	877.55	17-T2-0	789.47	17-T3-0	908.18	17-T4-0	731.99
18-T1-0	1022.23	18-T2-0	879.03	18-T3-0	1004.15	18-T4-0	946.64
19-T1-0	888.68	19-T2-0	793.57	19-T3-0	885.66	19-T4-0	1005.11
20-T1-0	862.10	20-T2-0	851.46	20-T3-0	1211.99	20-T4-0	731.76

Descriptive statistics of the data, outliers detection and exclusion - DPP IV enzymatic activities

	Mean (µU/mL)	S.D.	Median	Mean Dev.	S.E.M.	n
Control State	755.99	145.91	748.79	108.84	23.07	40
Treated State	754.22	174.62	730.13	143.03	27.61	40

Criteria: Outlier > 2.5 S.D.
Outliers: 20-T3-0, 2-T2-0
Statistical significance: n.s.

1.2.2.2 Serum enzymatic activities of DPP IV in volunteers - outliers excluded

Probes	Control [µU/mL]	Treated [µU/mL]
1-T1-0/1-T3-0	792.42	664.81
2-T1-0/2-T3-0	795.38	815.95
3-T1-0/3-T3-0	707.23	635.15
4-T1-0/4-T3-0	787.15	850.99
5-T1-0/5-T3-0	678.24	547.36
6-T1-0/6-T3-0	659.52	646.42
7-T1-0/7-T3-0	680.18	728.49
8-T1-0/8-T3-0	734.27	641.62
9-T1-0/9-T3-0	762.25	678.77
10-T1-0/10-T3-0	752.33	517.39
11-T1-0/11-T3-0	476.36	660.83
12-T1-0/12-T3-0	660.83	588.68
13-T1-0/13-T3-0	652.67	827.79
14-T1-0/14-T3-0	609.82	964.62
15-T1-0/15-T3-0	745.25	937.91
16-T1-0/16-T3-0	910.02	816.50
17-T1-0/17-T3-0	877.55	908.18
18-T1-0/18-T3-0	1022.23	1004.15
19-T1-0/19-T3-0	888.68	885.66
20-T1-0/20-T3-0	862.10	1211.99
1-T2-0/1-T4-0	1070.72	645.14
2-T2-0/2-T4-0	1168.63	661.79
3-T2-0/3-T4-0	650.21	628.58
4-T2-0/4-T4-0	881.58	665.06
5-T2-0/5-T4-0	493.32	541.59
6-T2-0/6-T4-0	658.09	586.96
7-T2-0/7-T4-0	741.51	572.89
8-T2-0/8-T4-0	607.82	732.73
9-T2-0/9-T4-0	701.28	770.40
10-T2-0/10-T4-0	496.83	498.41
11-T2-0/11-T4-0	586.30	651.84
12-T2-0/12-T4-0	769.46	810.87
13-T1-0/13-T3-0	691.42	1036.11
14-T2-0/14-T4-0	755.19	528.10
15-T2-0/15-T4-0	716.26	1108.53
16-T2-0/16-T4-0	882.79	780.83
17-T2-0/17-T4-0	789.47	731.99
18-T2-0/18-T4-0	879.03	946.64
19-T2-0/19-T4-0	793.57	1005.11
20-T2-0/20-T4-0	851.46	731.76

Descriptive statistical parameters of measured DPP IV enzymatic activities - outliers excluded

	Mean (µU/mL)	S.D.	Median	Mean Dev.	n
Control state (T1,T2)	745.41	131.35	745.25	100.54	39
Treated state (T3,T4)	742.47	160.12	728.49	132.85	39

Statistical significance: n.s.

4. Individual Changes in DPP IV enzymatic activities between T1-T3 and T2-T4 at time point 0 minute

For B-1.3.2.2 (Serum activities of DPP IV in healthy individuals, individual changes) after exclusion of the outliers the values 20-T1-0 and 2-T4-0 were combined and their ratio was calculated.

Individual change (%) in DPP IV activities between states T1-T3 and T2-T4:

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T1/1-T3	16.10	1-T2/1-T4	Outlier %	11-T1/11-T3	Outlier %	11-T2/11-T4	-11.18
2-T1/2-T3	-2.59	-	Outlier	12-T1/12-T3	10.92	12-T2/12-T4	-5.38
3-T1/3-T3	10.19	3-T2/3-T4	3.33	13-T1/13-T3	-26.83	13-T2/13-T4	Outlier %
4-T1/4-T3	-8.11	4-T2/4-T4	24.56	14-T1/14-T3	Outlier %	14-T2/14-T4	30.07
5-T1/5-T3	19.30	5-T2/5-T4	-9.79	15-T1/15-T3	-25.85	15-T2/17-T4	Outlier %
6-T1/6-T3	1.99	6-T2/6-T4	10.81	16-T1/16-T3	10.28	16-T2/16-T4	11.55
7-T1/7-T3	-7.10	7-T2/7-T4	22.74	17-T1/17-T3	-3.49	17-T2/17-T4	7.28
8-T1/8-T3	12.62	8-T2/8-T4	-20.55	18-T1/18-T3	1.77	18-T2/18-T4	-7.69
9-T1/9-T3	10.95	9-T2/9-T4	-9.86	19-T1/19-T3	0.34	19-T2/19-T4	-26.66
10-T1/10-T3	31.23	10-T2/10-T4	-0.32	20-T1/2-T4	23.24	20-T2/20-T4	14.06

11-T1-0/11-T3-0, 14-T1-0/14-T3-0, 1-T2-0/1-T4-0, 13-T2-0/13-T4-0, 15-T2-0/15-T4-0 - outlied ratios
20-T3-0, 2-T2-0 - outlied as previously described

	Control	Treated	Mean individual change between T1-T3 and T2-T4 at time point 0 minute: Decrease of 3.17 %, *Statistical significance: n.s.*
Mean (%)	100.00	96.83	
S.D. (%)	17.78	15.59	

1.3 Relaxin

The readouts were below the lowest limit of detection of the assay.

1.4 Adiponectin

1.4.1 Serum concentrations of adiponectin (ADN) before outliers exclusion

	ADN concentrations [µg/mL]							
Volunteer	T1-0	T1-120	T3-0	T3-120	T2-0	T2-120	T4-0	T4-120
1	5.83	5.53	6.22	5.74	6.04	7.16	6.66	6.3
2	7.16	6.69	7.24	7.12	5.65	6.46	6.71	7.08
3	3.62	3.34	3.12	2.98	4.68	4.05	3.71	4.27
4	10.34	10.48	11.48	9.35	9.1	10.48	10	11.68
5	8.65	11.25	10.51	10.76	13.12	11.24	15.27	13.18
6	6.71	8.17	7.79	7.69	9.08	7.72	11.53	10.86
7	7.72	8.61	8.13	8.2	11.04	7.06	7.72	8.00
8	6.64	7.61	6.82	6.89	7.52	7.57	10.32	7.47
9	6.58	6.62	6.84	6.26	5.75	7.33	5.54	8.57
10	5.95	5.15	5.84	4.93	6.12	5.99	4.89	4.56
11	6.61	6.3	6.28	5.88	6.17	6.68	5.54	6.15
12	7.71	8.84	6.65	7.76	6.87	8.3	9.23	8.19
13	9.09	10.6	8.36	10.53	9.78	8.96	11.99	13.1
14	7.48	7.35	7.08	6.84	9.65	6.98	8.34	6.74
15	5.16	7.94	4.47	6.00	6.31	6.1	4.92	6.08
16	14.7	15.03	16.87	17.22	14.21	16.53	13.94	16.14
17	10.28	10.84	11.67	13.1	9.67	9.5	10.92	12.85
18	9.09	8.01	6.24	7.08	7	6.98	7.09	6.99
19	5.93	5.59	6.11	6.68	6.47	5.76	5.93	6.45
20	9.75	12.2	10.34	10.47	11.22	11.31	9.64	11.47

Descriptive statistical parameters of adiponectin classic OGTT protocol data (n for each experimental state shown in the table), outliers detection and exclusion

Phase	Mean (µg/mL)	S.D.	Median	Mean Dev.	S.E.M.	n
T1-0	7.75	2.40	7.32	1.77	0.54	20
T1-120	8.31	2.78	7.98	2.14	0.62	20
T3-0	7.90	3.04	6.96	2.20	0.68	20
T3-120	8.07	3.16	7.10	2.31	0.71	20
T2-0	8.27	2.66	7.26	2.24	0.59	20
T2-120	8.11	2.72	7.25	1.96	0.61	20
T4-0	8.49	3.18	8.03	2.64	0.71	20
T4-120	8.81	3.29	7.74	2.76	0.74	20

Criteria: Outlier > 2.5 S.D.
Outliers: 16-T1-0, 16-T2-120, 16-T3-0, 16-T3-120
Statistical significance: n.s.

1.4.2 Serum concentrations of adiponectin (ADN) in volunteers - outliers excluded

	ADN concentrations [µg/mL]							
Volunteer	T1-0	T1-120	T3-0	T3-120	T2-0	T2-120	T4-0	T4-120
1	5.83	5.53	6.22	5.74	6.04	7.16	6.66	6.3
2	7.16	6.69	7.24	7.12	5.65	6.46	6.71	7.08
3	3.62	3.34	3.12	2.98	4.68	4.05	3.71	4.27
4	10.34	10.48	11.48	9.35	9.1	10.48	10	11.68
5	8.65	11.25	10.51	10.76	13.12	11.24	15.27	13.18
6	6.71	8.17	7.79	7.69	9.08	7.72	11.53	10.86
7	7.72	8.61	8.13	8.2	11.04	7.06	7.72	8
8	6.64	7.61	6.82	6.89	7.52	7.57	10.32	7.47
9	6.58	6.62	6.84	6.26	5.75	7.33	5.54	8.57
10	5.95	5.15	5.84	4.93	6.12	5.99	4.89	4.56
11	6.61	6.3	6.28	5.88	6.17	6.68	5.54	6.15
12	7.71	8.84	6.65	7.76	6.87	8.3	9.23	8.19
13	9.09	10.6	8.36	10.53	9.78	8.96	11.99	13.1
14	7.48	7.35	7.08	6.84	9.65	6.98	8.34	6.74
15	5.16	7.94	4.47	6	6.31	6.1	4.92	6.08
16	Outlier	15.03	Outlier	Outlier	14.21	Outlier	13.94	16.14
17	10.28	10.84	11.67	13.1	9.67	9.5	10.92	12.85
18	9.09	8.01	6.24	7.08	7	6.98	7.09	6.99
19	5.93	5.59	6.11	6.68	6.47	5.76	5.93	6.45
20	9.75	12.2	10.34	10.47	11.22	11.31	9.64	11.47

Descriptive statistical parameters of adiponectin classic and rice OGTT protocol data (n for each experimental state shown in the table) - outliers excluded

Phase	Mean (µg/mL)	S.D.	Median	Mean Dev.	S.E.M.	n
T1-0	7.38	1.8	7.16	1.44	0.41	19
T1-120	8.31	2.78	7.98	2.14	0.62	20
T3-0	7.43	2.25	6.84	1.71	0.52	19
T3-120	7.59	2.38	7.08	1.8	0.55	19
T2-0	8.27	2.66	7.26	2.24	0.59	20
T2-120	7.66	1.91	7.16	1.46	0.44	19
T4-0	8.49	3.18	8.03	2.64	0.71	20
T4-120	8.81	3.29	7.74	2.76	0.74	20

Statistical significance: n.s.

Individual changes in ADN concentrations

A. Individual change (%) in ADN concentrations between states T1-T3 and T2-T4 - 0 minute:

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T1/ 1-T3	-6.69	1-T2-0/ 1-T4-0	-10.26	11-T1-0/ 11-T3-0	4.99	11-T2-0/ 11-T4-0	10.21
2-T1/ 2-T3	-1.12	2-T2-0/ 2-T4-0	-18.76	12-T1-0/ 12-T3-0	13.75	12-T2-0/ 12-T4-0	-34.35
3-T1/ 3-T3	13.81	3-T2-0/ 3-T4-0	20.73	13-T1-0/ 13-T3-0	8.03	13-T2-0/ 13-T4-0	-22.60
4-T1/ 4-T3	-11.03	4-T2-0/ 4-T4-0	-9.89	14-T1-0/ 14-T3-0	5.35	14-T2-0/ 14-T4-0	13.58
5-T1/ 5-T3	-21.50	5-T2-0/ 5-T4-0	-16.39	15-T1-0/ 15-T3-0	13.37	15-T2-0/ 15-T4-0	22.03
6-T1/ 6-T3	-16.10	6-T2-0/ 6-T4-0	-26.98	16-T1-0/ 16-T3-0	Outlier	16-T2-0/ 16-T4-0	1.90
7-T1/ 7-T3	-5.31	7-T2-0/ 7-T4-0	30.07	17-T1-0/ 17-T3-0	-13.52	17-T2-0/ 17-T4-0	-12.93
8-T1/ 8-T3	-2.71	8-T2-0/ 8-T4-0	Outlier %	18-T1-0/ 18-T3-0	31.35	18-T2-0/ 18-T4-0	-1.29
9-T1/ 9-T3	-3.95	9-T2-0/ 9-T4-0	3.65	19-T1-0/ 19-T3-0	-3.04	19-T2-0/ 19-T4-0	8.35
10-T1/ 10-T3	1.85	10-T2-0/ 10-T4-0	20.10	20-T1-0/ 20-T3-0	-6.05	20-T2-0/ 20-T4-0	14.08

16-T1-0/16-T3-0 - outlied as described previously
8-T2-0/8-T4-0 - outlied ratio

	Control State (T1-0, T2-0)	Treated State (T3-0, T4-0)	n= 38 Mean Individual Change between T1-T3 and T2-T4 at time point 0 minute: 0.19 %, *Statistical significance: n.s.*
Mean (%)	100.00	100.19	
S.D. (%)	29.61	15.63	

B. Individual changes of adiponectin upon OGTT (compared to baseline)

B1. Individual changes between T1-0 and T1-120

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T1-0/ 1-T1-120	5.15	6-T1-0/ 6-T1-120	-21.76	11-T1-0/ 11-T1-120	4.69	16-T1-0/ 16-T1-120	Outlier
2-T1-0/ 2-T1-120	6.56	7-T1-0/ 7-T1-120	-11.53	12-T1-0/ 12-T1-120	-14.66	17-T1-0/ 17-T1-120	-5.45
3-T1-0/ 3-T1-120	7.73	8-T1-0/ 8-T1-120	-14.61	13-T1-0/ 13-T1-120	-16.61	18-T1-0/ 18-T1-120	11.88
4-T1-0/ 4-T1-120	-1.35	9-T1-0/ 9-T1-120	-0.61	14-T1-0/ 14-T1-120	1.74	19-T1-0/ 19-T1-120	5.73
5-T1-0/ 5-T1-120	Outlier %	10-T1-0/ 10-T1-120	13.45	15-T1-0/ 15-T1-120	Outlier %	20-T1-0/ 20-T1-120	-25.13

16-T1-0/16-T1-120 - outlied as described previously
5-T1-0/5-T1-120, 15-T1-0/15-T1-120 - outlied ratios

	T1-0	T1-120	n= 17, Mean Individual Change between T1-0 and T1-120: Increase of 3.22 %
Mean (%)	100.00	103.22	
S.D. (%)	24.18	12.01	

B2. Individual changes between T2-0 and T2-120

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T2-0/ 1-T2-120	-18.54	6-T2-0/ 6-T2-120	14.98	11-T2-0/ 11-T2-120	-8.27	16-T2-0/ 16-T2-120	Outlier
2-T2-0/ 2-T2-120	-14.34	7-T2-0/ 7-T2-120	Outlier %	12-T2-0/ 12-T2-120	-20.82	17-T2-0/ 17-T2-120	1.76
3-T2-0/ 3-T2-120	13.46	8-T2-0/ 8-T2-120	-0.66	13-T2-0/ 13-T2-120	8.38	18-T2-0/ 18-T2-120	0.29
4-T2-0/ 4-T2-120	-15.16	9-T2-0/ 9-T2-120	-27.48	14-T2-0/ 14-T2-120	27.67	19-T2-0/ 19-T2-120	10.97
5-T2-0/ 5-T2-120	14.33	10-T2-0/ 10-T2-120	2.12	15-T2-0/ 15-T2-120	3.33	20-T2-0/ 20-T2-120	-0.80

16-T1-0/16-T1-120 - outlied as described previously
7-T2-0/7-T2-120 - outlied ratio

	T2-0	T2-120	n= 18, Mean Individual Change between T2-0 and T2-120: 0.49 % *Statistical significance: n.s.*
Mean (%)	100.00	100.49	
S.D. (%)	29.02	14.59	

B3. Individual changes between T3-0 and T3-120

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T3-0/ 1-T3-120	7.72	6-T3-0/ 6-T3-120	1.28	11-T3-0/ 11-T3-120	6.37	16-T3-0/ 16-T3-120	Outlier
2-T3-0/ 2-T3-120	1.66	7-T3-0/ 7-T3-120	-0.86	12-T3-0/ 12-T3-120	-16.69	17-T3-0/ 17-T3-120	-12.25
3-T3-0/ 3-T3-120	4.49	8-T3-0/ 8-T3-120	-1.03	13-T3-0/ 13-T3-120	Outlier %	18-T3-0/ 18-T3-120	-13.46
4-T3-0/ 4-T3-120	18.55	9-T3-0/ 9-T3-120	8.48	14-T3-0/ 14-T3-120	3.39	19-T3-0/ 19-T3-120	-9.33
5-T3-0/ 5-T3-120	-2.38	10-T3-0/ 10-T3-120	15.58	15-T3-0/ 15-T3-120	Outlier %	20-T3-0/ 20-T3-120	-1.26

16-T3-0/16-T3-120 -outlied as described previously
13-T3-0/13-T3-120, 15-T3-0/15-T3-120 - outlied ratios

	T3-0	T3-120	n= 17, Mean Individual Change between T3-0 and T3-120: Decrease of 0.60 % *Statistical significance: n.s.*	
Mean (%)	100.00	99.40		
S.D. (%)	29.84	9.67		

B4. Individual changes between T4-0 and T4-120

Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)	Samples	Individual Change (%)
1-T4-0/ 1-T4-120	5.41	6-T4-0/ 6-T4-120	5.81	11-T4-0/ 11-T4-120	-11.01	16-T4-0/ 16-T4-120	-15.78
2-T4-0/ 2-T4-120	-5.51	7-T4-0/ 7-T4-120	-3.63	12-T4-0/ 12-T4-120	11.27	17-T4-0/ 17-T4-120	-17.67
3-T4-0/ 3-T4-120	-15.09	8-T4-0/ 8-T4-120	Outlier %	13-T4-0/ 13-T4-120	-9.26	18-T4-0/ 18-T4-120	1.41
4-T4-0/ 4-T4-120	-16.80	9-T4-0/ 9-T4-120	Outlier %	14-T4-0/ 14-T4-120	19.18	19-T4-0/ 19-T4-120	-8.77
5-T4-0/ 5-T4-120	13.69	10-T4-0/ 10-T4-120	6.75	15-T4-0/ 15-T4-120	-23.58	20-T4-0/ 20-T4-120	-18.98

8-T4-0/8-T4-120, 9-T4-0/9-T4-120 - outlied ratios

	T4-0	T4-120	n= 18, Mean Individual Change between T4-0 and T4-120: Increase of 4.59 % *Statistical significance: n.s.*
Mean (%)	100	104.588	
S.D. (%)	38.0299	12.6705	

2 Section 2 – Akt

2.1 Pilot study – normalized values

No.	Control		Resveratrol, 30 min (%)	Quercetin, 30 min (%)
	Absolute	Norm. (%)		
1.	1.28	100.00	58.90	40.07
2.	1.65	100.00	55.03	31.44
3.	1.51	100.00	63.26	71.37
4.	1.11	100.00	71.65	-
Mean (%)	100.00		62.21	47.63
S.D. (%)	17.36		7.14	21.01
Mean Inhibition (%)	0.00		37.79	52.37
n	4		4	3

No.	Control		Resveratrol, 15 min (%)
	Absolute	Norm. (%)	
1.	1.00	100.00	30.93
2.	1.08	100.00	53.97
3.	1.00	100.00	72.34
4.	1.05	100.00	82.53
Mean (%)	100.00		60.11
S.D. (%)	3.82		23.06
Mean Inhibition (%)	0.00		39.89
n	4		4

2.2 Primary screening – normalized values

2.2.1 pAkt Ser473

[%]	Negative control	Quercetin	Resveratrol	Apigenin	Luteolin
Mean	100.00	59.54	74.15	77.43	70.04
Inhibition	0.00	40.46	25.85	22.57	29.96
S.D.	9.73	4.83	5.72	10.30	11.06
n	29	5	6	6	6

[%]	(+)-Catechin	Genistein	Taxifolin	3-MS	Positive control
Mean	109.51	93.23	99.44	91.68	8.16
Inhibition	-9.51	6.67	0.56	8.32	91.84
S.D.	15.35	18.98	17.96	14.16	2.18
n	4	6	5	3	3

3-MS= 3,4’,5-Trimethoxy-trans-stilbene; Positive control – serum free starving overnight

2.2.2 pAkt Thr308

[%]	Negative control	Quercetin	Resveratrol	Apigenin	Luteolin
Mean	100.00	64.29	68.22	71.90	73.10
Inhibition	0.00	35.71	31.78	28.10	26.90
S.D.	9.08	3.73	13.09	10.21	10.33
n	24	3	4	6	6

[%]	(+)-Catechin	Genistein	Taxifolin	3-MS	Positive control
Mean	98.64	89.95	100.31	94.42	11.36
Inhibition	1.36	10.05	-0.31	5.58	88.64
S.D.	10.51	14.59	10.21	21.41	5.33
n	4	4	5	3	3

3-MS= 3,4',5-Trimethoxy-trans-stilbene; Positive control – serum free starving overnight

2.3 Secondary screening (pAkt Ser473) – normalized values

Group I: Substances with pronounced inhibitory potential (> 20 %)

[%]	Negative control	Quercetin	Resveratrol	Apigenin	Luteolin	6-HF
Mean	100.00	64.29	73.96	68.21	55.69	73.14
Inhibition	0.00	35.71	26.04	31.79	44.31	26.86
S.D.	5.19	8.33	5.58	6.16	17.95	7.78
n	37	6	5	5	6	2

[%]	6-MF	Chrysin	7,8-DHF	Urolithin A	Fisetin	Flavone
Mean	75.11	78.30	79.68	64.72	71.91	77.76
Inhibition	24.89	21.70	20.32	35.28	28.09	22.24
S.D.	10.29	N/A	N/A	11.80	9.09	2.28
n	2	1	1	6	3	2

6-HF= 6-Hydroxyflavone; 6-MF= 6-Methoxyflavone; 7,8-DHF= 7,8-Dihydroxyflavone

Group II: Substances with inhibitory potential between 10 % and 20 %

[%]	Negative control	Pinostilbene	7-MF	Baicalein	Ellagic Acid
Mean	100.00	80.66	81.61	81.82	86.02
Inhibition	0.00	19.34	18.39	18.18	13.98
S.D.	5.19	14.45	N/A	N/A	5.64
n	37	2	1	1	2

[%]	Urolithin D	(-)-GCG	3-MF	3,4'-DHF	Myricetin	Kaempferol
Mean	88.83	87.77	87.15	89.31	81.09	82.98
Inhibition	11.17	12.23	12.85	10.69	18.91	17.02
S.D.	N/A	N/A	N/A	N/A	N/A	11.81
n	1	1	1	1	1	3

7-MF= 7-Methoxyflavone; (-)-GCG= (-)-Gallocatechin gallate; 3-MF= 3-Methoxyflavone; 3,4'-DHF= 3,4'-Dihydroxyflavone

Group III: Substances with slight inhibitory potential between 5 % and 10 %

[%]	Negative control	Genistein	Taxifolin	3-HF	Urolithin B	Morin
Mean	100.00	90.95	91.31	93.66	94.78	90.31
Inhibition	0.00	9.05	8.69	6.34	5.22	9.69
S.D.	5.19	N/A	N/A	4.22	N/A	N/A
n	37	1	1	2	1	1

3-HF= 3-Hydroxyflavone;

Group IV: Substances with no inhibitory potential (Not hits)

[%]	Negative control	Caffeic acid	M1	Urolithin C	EPI	EGCG
Mean	100.00	99.01	100.33	100.08	104.23	102.03
Inhibition	0.00	0.99	-0.33	-0.08	-4.23	-2.03
S.D.	5.19	N/A	N/A	N/A	N/A	N/A
n	37	1	1	1	1	1

[%]	3-H-4'-MF	Wogonoside	Pterostilbene	3-MS	Piacetannol	Chlorogenic Acid
Mean	97.23	105.02	105.96	100.93	102.73	96.41
Inhibition	2.77	-5.02	-5.96	-0.93	-2.73	3.59
S.D.	N/A	N/A	1.59	9.99	N/A	N/A
n	1	1	2	2	1	1

EPI= Epigallocatechin; EGCG= Epigallocatechin gallate; 3-H-4'-MF= 3-Hydroxy-4'-Methoxyflavone; 3-MS= 3,4',5-Trimethoxy-trans-stilbene;

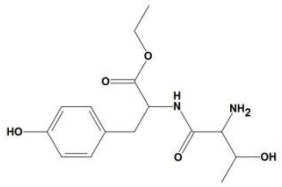
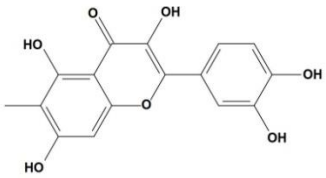
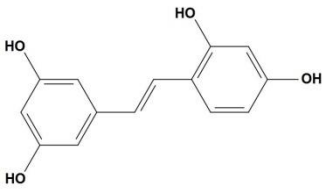
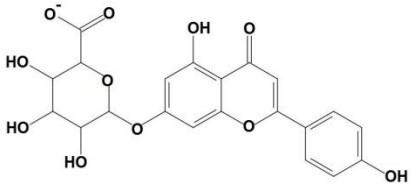
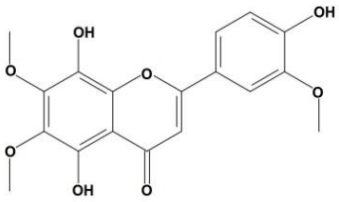
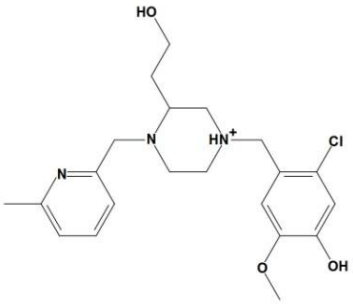
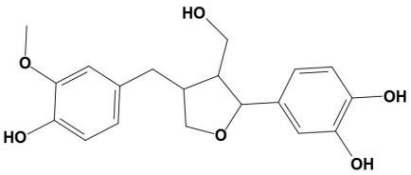
[%]	(+)-Catechin	(-)-Epicatechin	Naringenin	M2	Ferulic acid	Baicalin	Vitexin
Mean	105.10	110,73	107,32	109.35	108.32	110.85	108,51
Inhibition	-5.10	-10,73	-7,32	-9.35	-8.32	-10.85	-8,51
S.D.	17.14	4.90	N/A	8.90	21.35	N/A	N/A
n	4	3	1	3	3	1	1

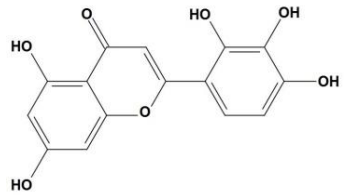
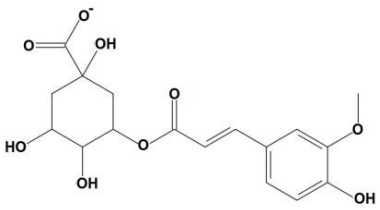
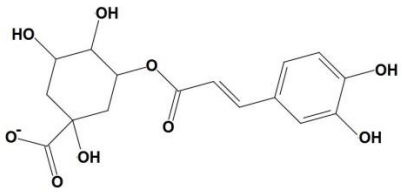
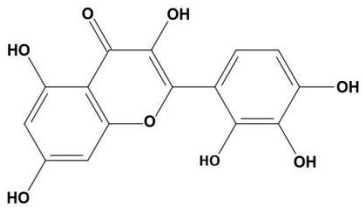
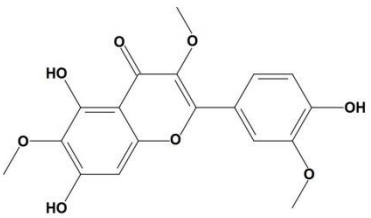
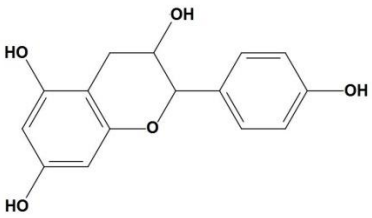
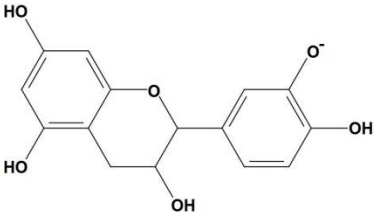
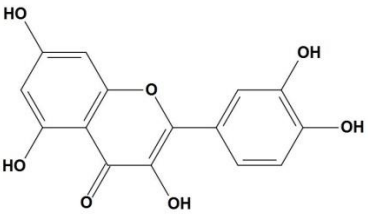
2.4 Metformin's effects on pAkt Ser473 in EA.hy926, normalized values

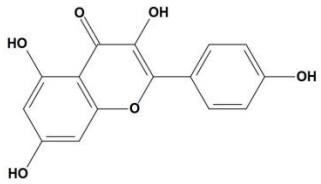
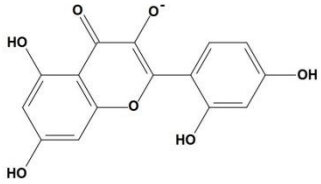
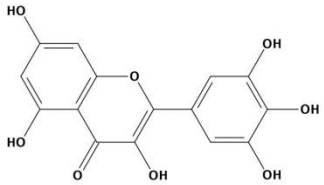
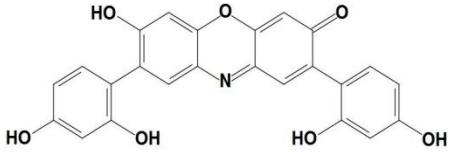
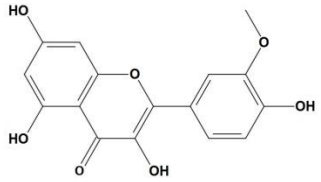
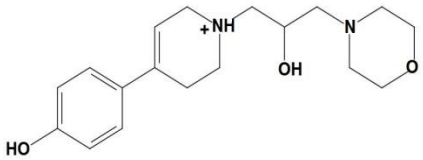
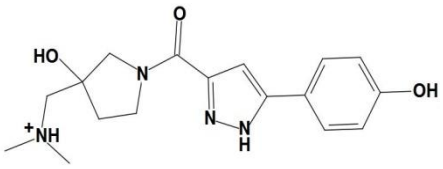
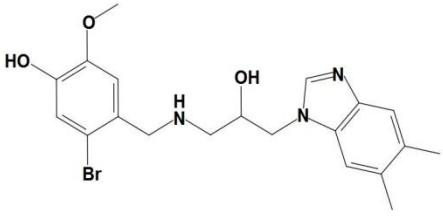
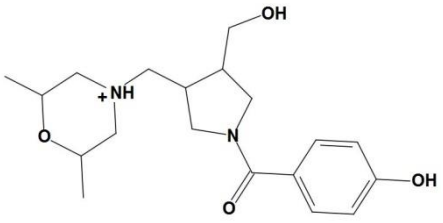
No.	Negative controls	Metformin, 20 uM, 2.5 h
1.	100.00	97.94
2.	100.00	87.14
3.	105.59	96.18
4.	100.00	97.15
5.	100.00	105.29
6.	101.59	96.27
	Negative controls	Metformin, 20 uM, 2.5 h
mean	100.00	95.52
S.D.	2.22	5.72

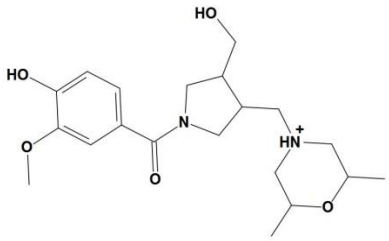
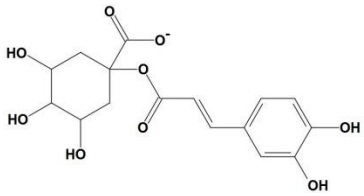
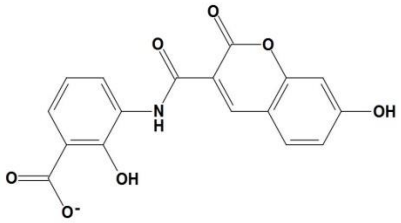
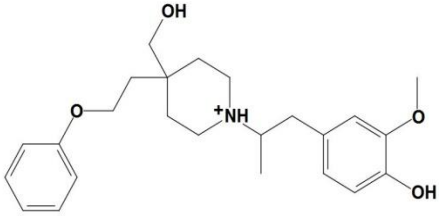
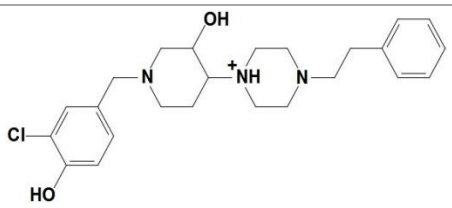
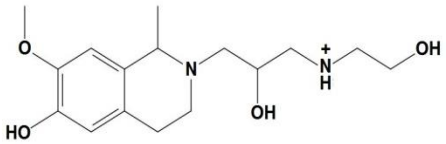
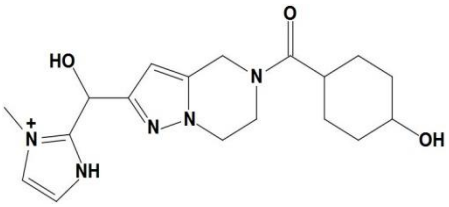
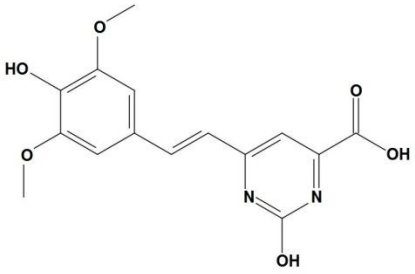
3 Section 3 – Virtual screening

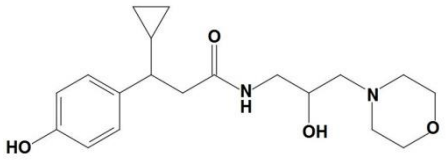
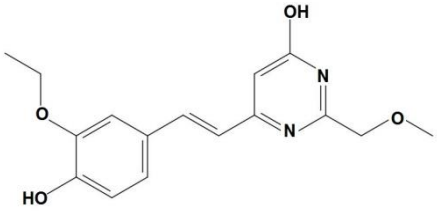
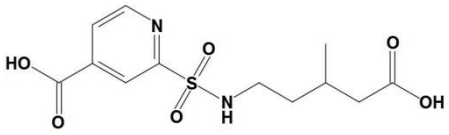
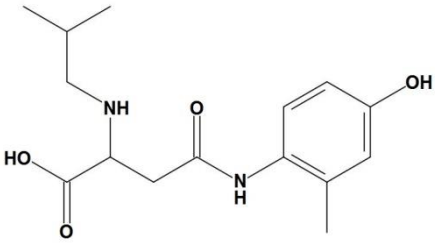
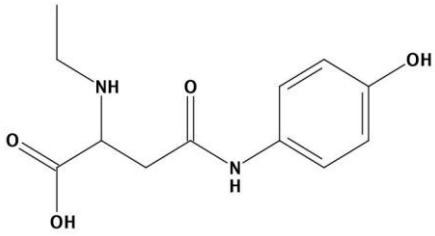
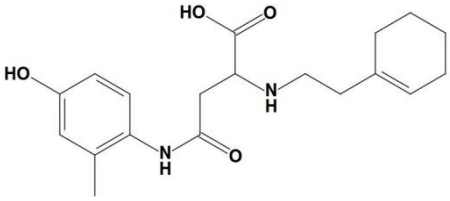
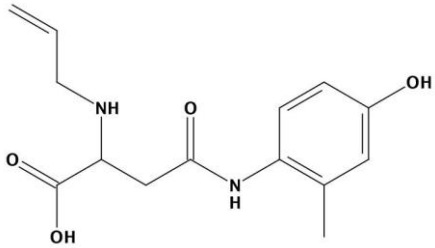
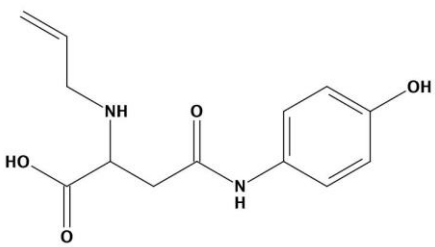
3.1. Virtual screening hits; Matches of exclusive (A) and planar (B) models:

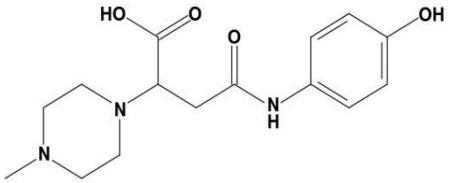
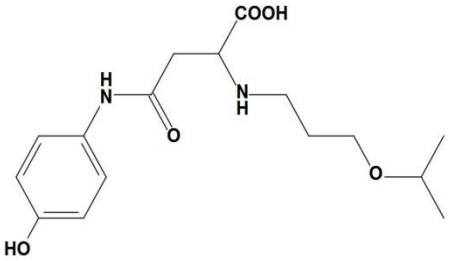
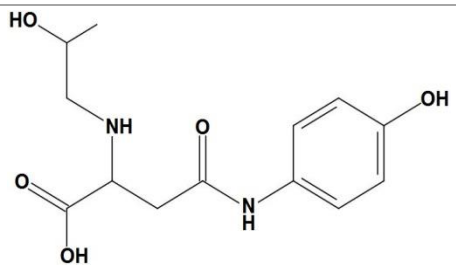
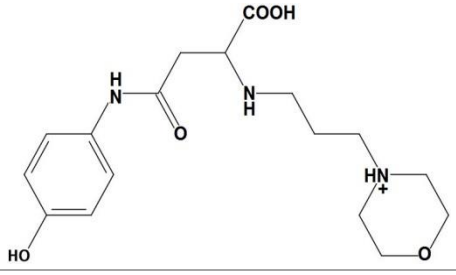
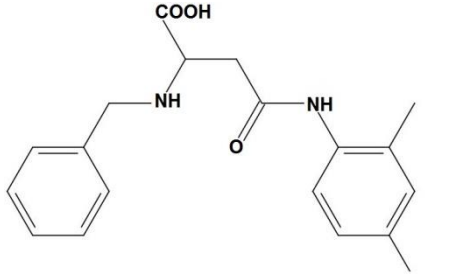
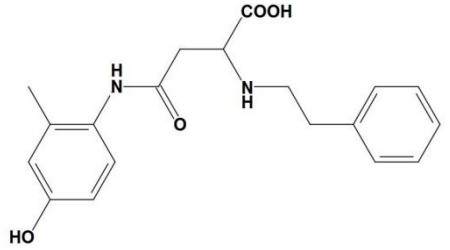
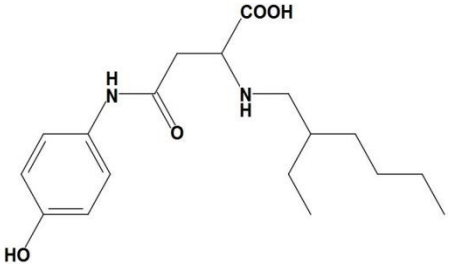
No	Vendor code	Chemical structure of the hits	PM
1	2746422		A
2	5756759		A, B
3	5756987		A
4	6861214		A
5	12946571		A
6	20683870		A, B
7	23914983		A

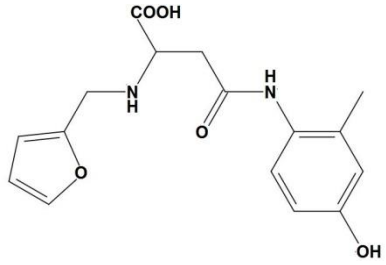
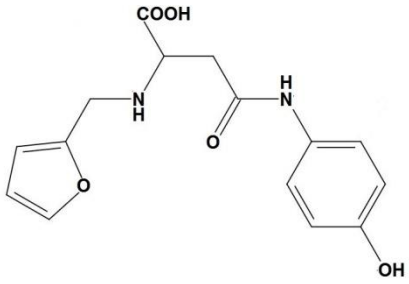
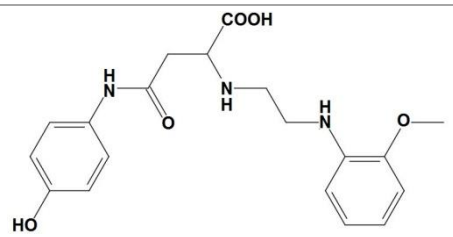
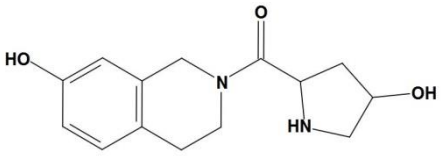
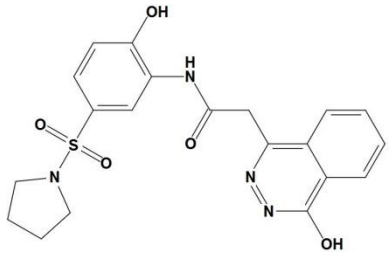
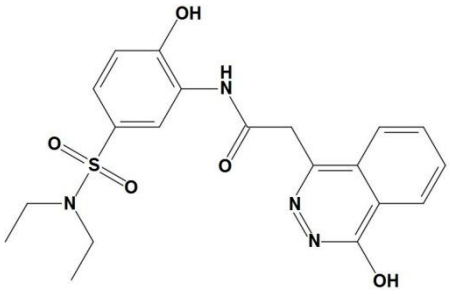
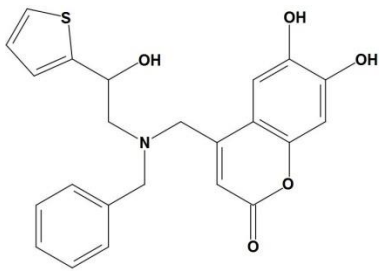
No	Vendor code	Chemical structure of the hits	PM
8	23915227		A, B
9	25719106		A
10	25719120 (SW02476)		A
11	28295268		A, B
12	31705442		A
13	31705716		A
14	489431		A
15	497873 (Z57176222)		A, B

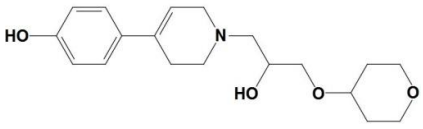
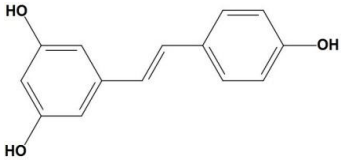
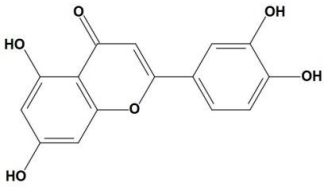
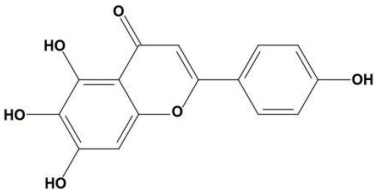
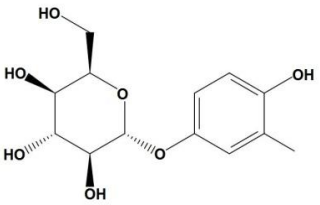
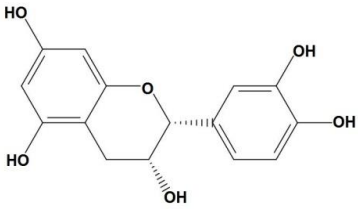
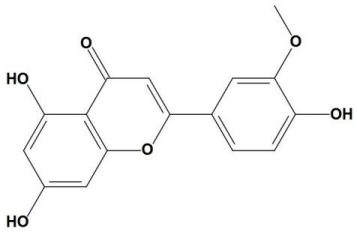
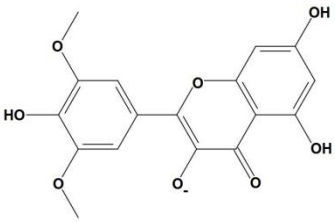
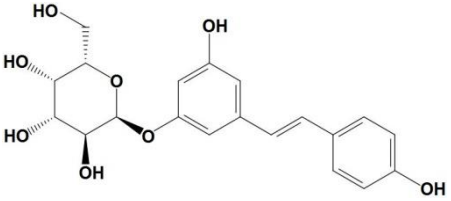
No	Vendor code	Chemical structure of the hits	PM
16	524530		A, B
17	529291		A, B
18	529337 (SW02044) (DB02375)		A, B
19	716311		A
20	1935868 (SW02009)		A, B
21	33300818 (Z197493618)		A, B
22	43173679		A
23	43378426		A
24	44734711		A

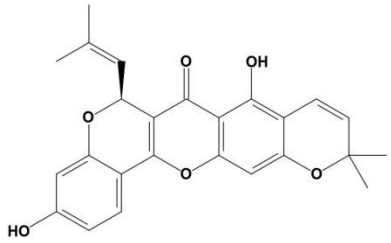
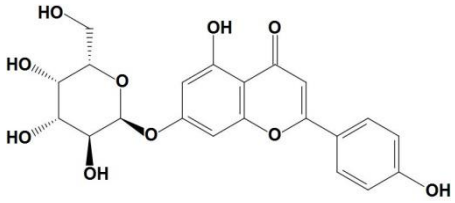
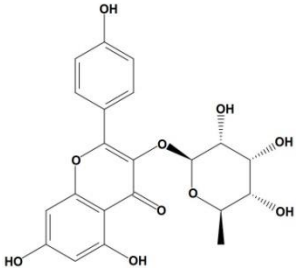
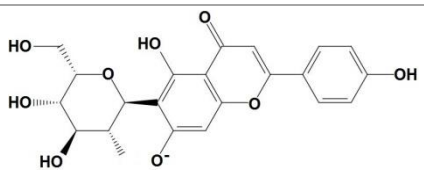
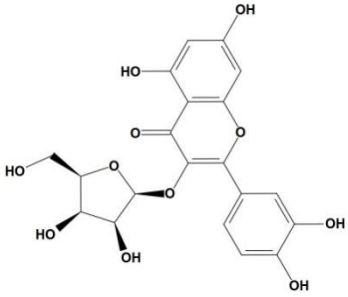
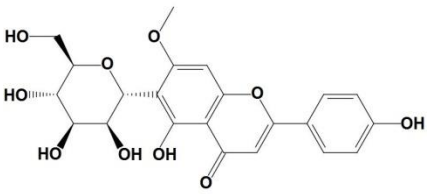
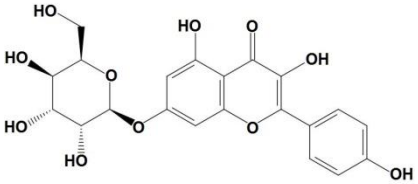
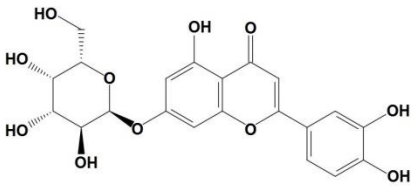
No	Vendor code	Chemical structure of the hits	PM
25	44746389		A
26	44843457		A
27	49401106		A, B
28	20412015		B
29	26016190		B
30	26407805		B
31	55663566		B
32	Z1567937963		A, B

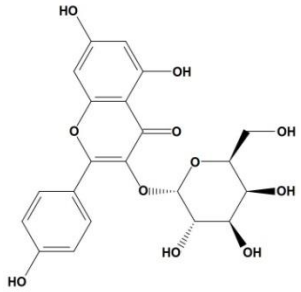
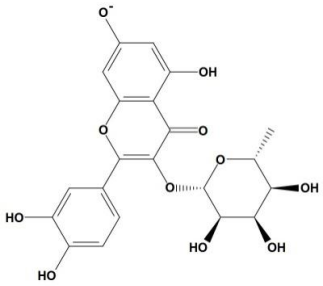
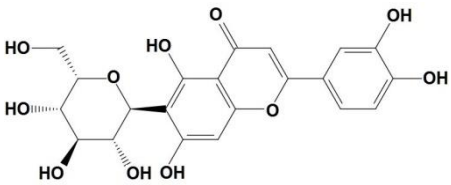
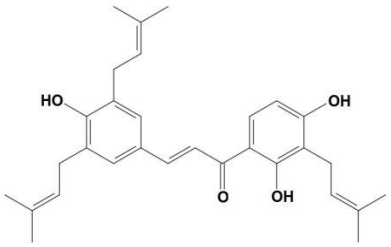
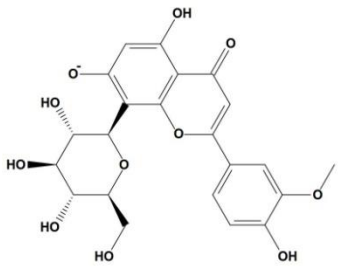
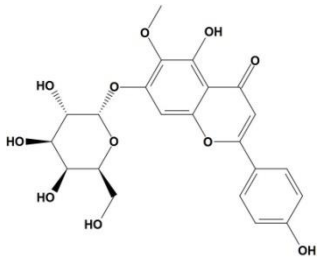
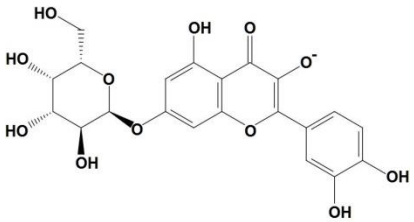
No	Vendor code	Chemical structure of the hits	PM
33	Z2329004358		A
34	Z2752916184		A, B
35	Z2936916050		A
36	F2721-0733		A, B
37	F2721-0110		A, B
38	F2721-0728		A, B
39	F2721-0737		A, B
40	F1124-0003		A, B

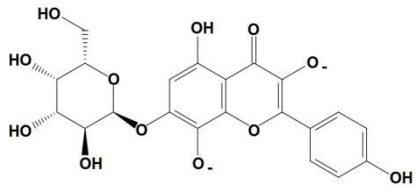
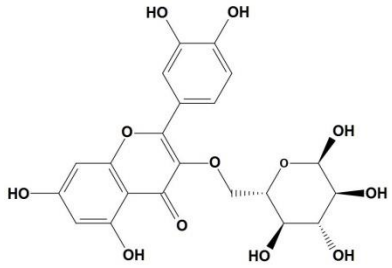
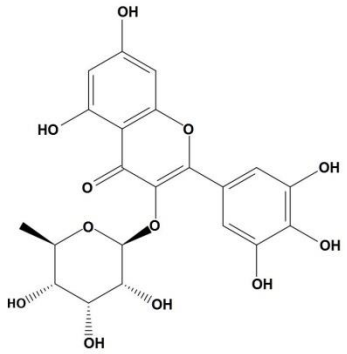
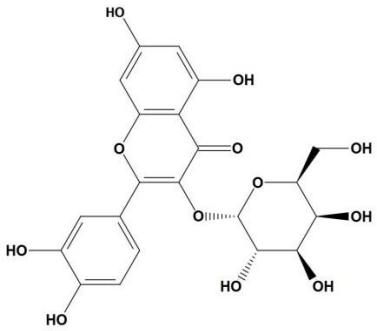
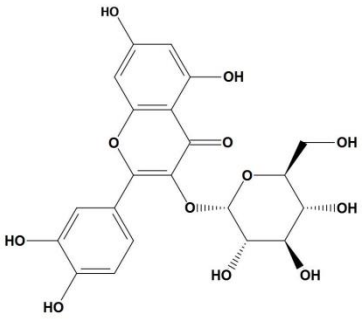
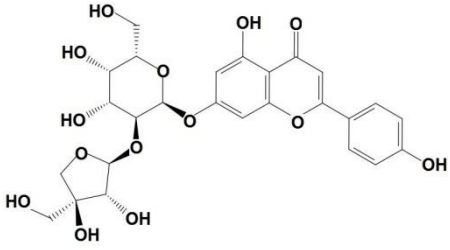
No	Vendor code	Chemical structure of the hits	PM
41	F1278-0316		A
42	F2721-0115		A, B
43	F2721-0112		A, B
44	F2721-0109		A, B
45	F2721-0726		A, B
46	F2721-0727		A, B
47	F2721-0111		A, B

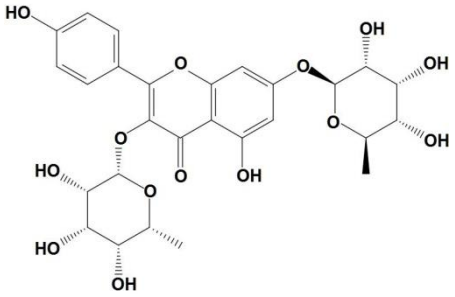
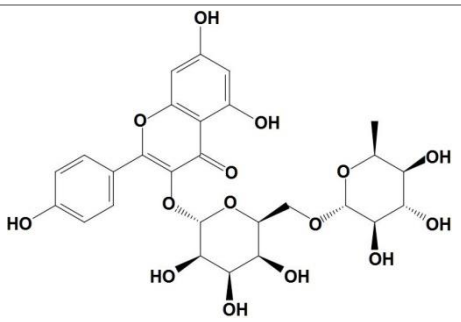
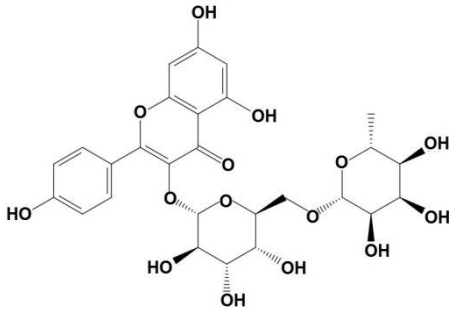
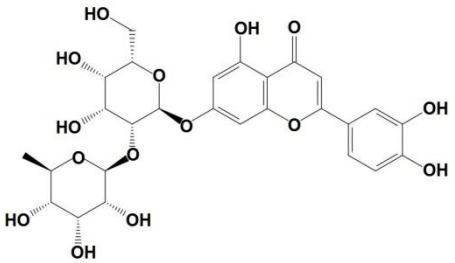
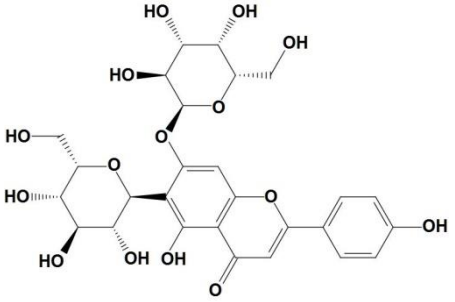
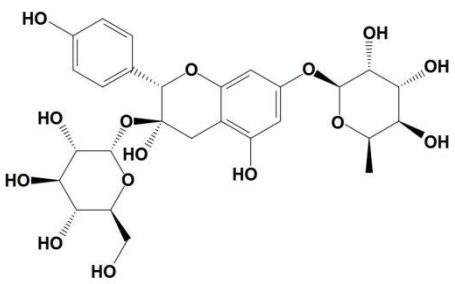
No	Vendor code	Chemical structure of the hits	PM
48	F2721-0729		A, B
49	F1278-0303		A, B
50	F2721-0118		A, B
51	Z2573054171		B
52	Z184743814		A
53	Z198619234		A
54	Z1621797376		A

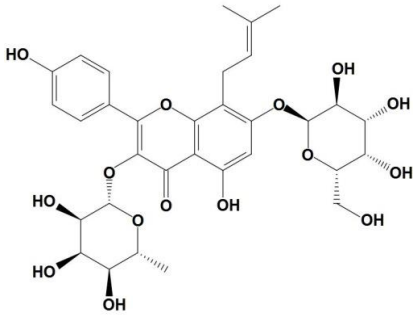
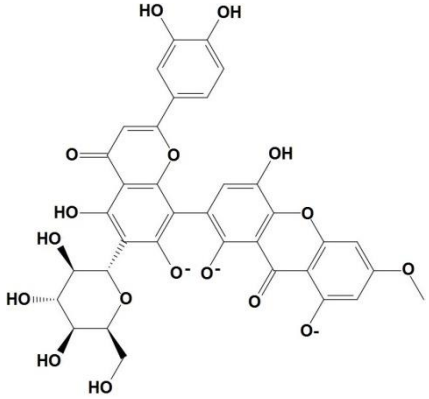
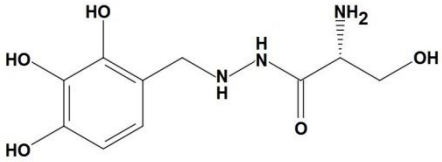
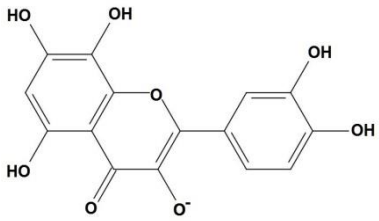
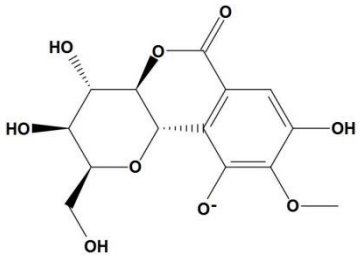
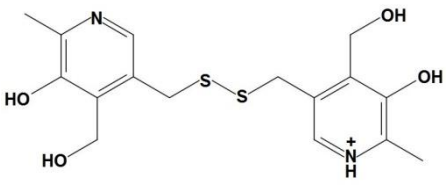
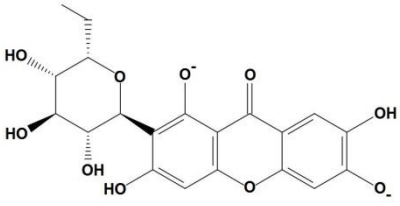
No	Vendor code	Chemical structure of the hits	PM
55	Z1393075894		A
56	SW00982		A
57	SW01592		A, B
56	SW01593		A, B
57	SW01608		A,B
58	SW01668		A,B
59	SW01797		A,B
60	SW02380		A, B
61	SW02899		A

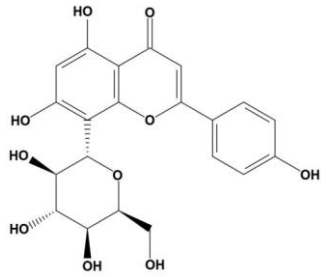
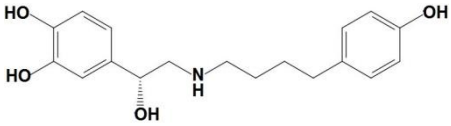
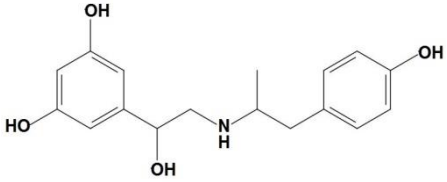
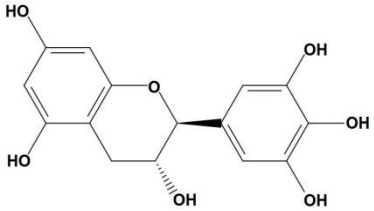
No	Vendor code	Chemical structure of the hits	PM
62	SW03154		A, B
63	SW03270		A, B
64	SW03269		A, B
65	SW03275		A, B
66	SW03290		A
67	SW03366		A
68	SW03369		A, B
69	SW03378		A, B

No	Vendor code	Chemical structure of the hits	PM
70	SW03381		A
71	SW03376		A, B
72	SW03380		A, B
73	SW03465		A, B
74	SW03470		A, B
75	SW03472		A
76	SW03477		A, B

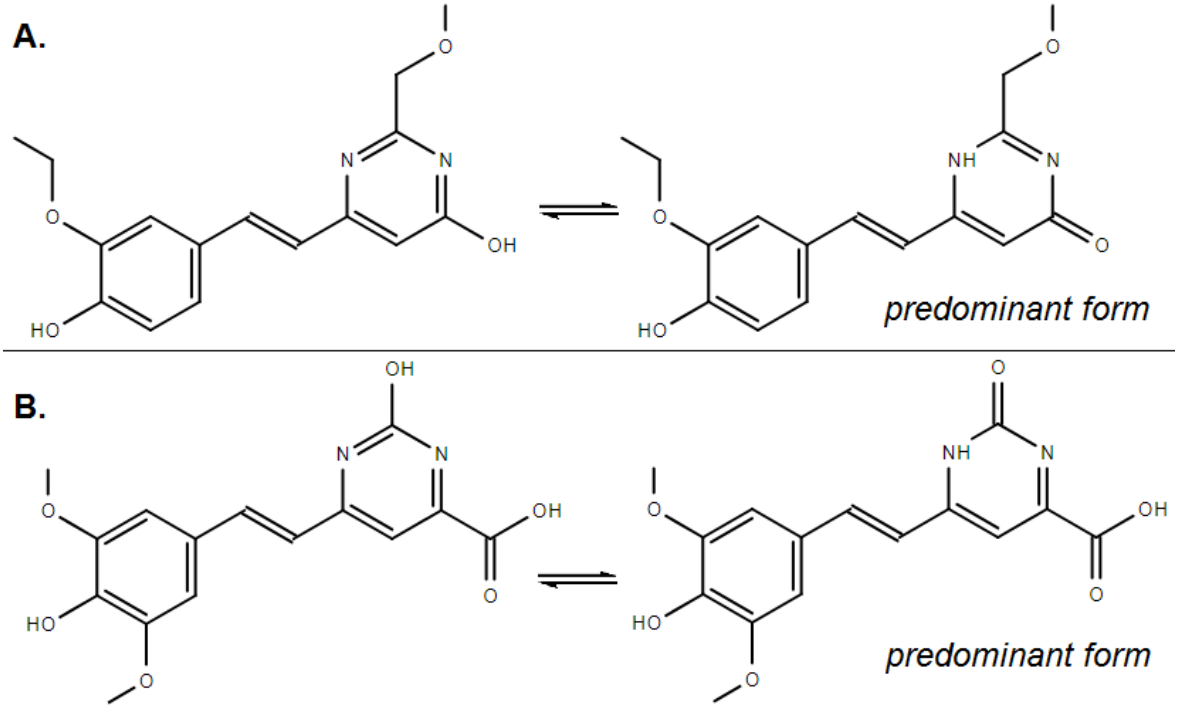
No	Vendor code	Chemical structure of the hits	PM
77	SW03478		A, B
78	SW03489		A, B
79	SW03488		A
80	SW03490		A
81	SW03491		A
82	SW03848		A, B

No	Vendor code	Chemical structure of the hits	PM
83	SW03878		A
84	SW03923		A
85	SW03922		A, B
86	SW03921		A, B
87	SW03925		A
88	SW03935		A, B

No	Vendor code	Chemical structure of the hits	PM
89	SW04037		A
90	SW04086		A, B
91	SW01285		B
92	SW02045		B
93	SW02190		B
94	SW02667		B
95	SW03179		B

No	Vendor code	Chemical structure of the hits	PM
96	SW03271		B
97	DB01102		A
98	DB01288		A
99	DB03823		A

3.2. Tautomerization of two of the hits Compound 1 (A) and Compound 2 (B) from Table 8, B-2.5.7 defined by MoKa software.



Proof of concept – in vitro test of virtual screening hits

	Negative control	Compound 1	Compound 2	Compound 3	Compound 4
mean	100.00	99.46	98.34	108.42	105.87
S.D.	7.29	16.16	15.43	22.11	12.88
M.D.	5.17	12.89	12.62	18.36	10.49
median	100.73	100.60	103.65	98.37	103.10
n	15	5	6	5	10

4 Section 4 – DPP IV activity and expression

4.1 DPP IV activity inhibition by polyphenols

No.	Compound	Mean Inhibition (%)	Mean Dev (%)	n	Notes
1.	Quercetin	9.42	2.40	4	** p< 0.01
2.	Catechin	5.63	0.62	3	n.s.
3.	Resveratrol	5.65	1.28	3	n.s.
4.	Elagic Acid	3.74	1.75	4	n.s.
5.	Urolithin A	2.60	3.61	3	n.s.
6.	Urolithin B	0.61	0.57	2	n.s.
7.	Urolithin C	3.45	1.60	3	n.s.
8.	Urolithin D	6.15	0.0	1	n.s.
9.	Apigenin	6.04	3.03	4	n.s.
10.	Luteolin	6.21	0.56	2	n.s.
11.	Taxifolin	0.15	0.0	1	n.s.
12.	Naringenin	2.08	0.0	1	n.s.
13.	Fisetin	3.52	0.0	1	n.s.
14.	Rutin	0.54	0.0	1	n.s.
15.	Kaempferol	5.79	0.0	1	n.s.
16.	Morine	5.26	0.0	1	n.s.
17.	Baicalein	7.99	0.0	1	n.s.
18.	7,8-Dihidroxyflavone	7.02	0.0	1	n.s.
19.	M1 Valerolactone	2.89	0.0	1	n.s.
20.	Pinostilbene	2.86	0.0	1	n.s.
21.	Pterostilbene	1.82	0.0	1	n.s.
22.	3,4,5-trimethoxy-trans-stilbene	1.32	0.0	1	n.s.
23.	Picetannol	5.60	0.0	1	n.s.
24.	Isorhapontigenin	5.04	0.0	1	n.s.
25.	Oxyresveratrol	3.67	0.0	1	n.s.
26.	Piceid	3.39	0.0	1	n.s.
27.	Caffeic Acid	4.70	0.0	1	n.s.
28.	Chrysin	0.00	0.0	1	n.s.
29.	Genistein	-1.80	0.0	1	Assay interaction possible: intrinsic fluorescence
30.	3-Hydroxyflavone	-5.29	0.0	1	
31.	3-Hydroxy-4'-methoxyflavone	-6.93	0.0	1	
32.	6-Hydroxyflavone	-21.00	0.0	1	
33.	6-Methoxyflavone	-11.15	0.0	1	
34.	7-Methoxyflavone	-10.20	0.0	1	
35.	Myricetin	15.32	0.0	1	Assay interaction possible: sudden color change

5 Section 5 – Fluorescent interactions

Table 1.1.: Descriptive statistical parameters of normalized fluorescent values of blanks, quercetin, urolithin A, urolithin B and 6-MF in assay buffer under the tested experimental conditions (n= 8).

	0 min, **no AMC**, assay buffer				30 min, **no AMC**, assay buffer			
	Mean [%]	S.D. [%]	Median [%]	p	Mean [%]	S.D. [%]	Median [%]	p
Blank	100.00	5.63	102.85	-	100.00	5.16	100.92	-
Quercetin	99.86	4.13	99.53	> 0.05	99.95	2.26	99.86	> 0.05
Urolithin A	102.77	2.73	101.92	> 0.05	104.35	1.29	104.39	> 0.05
Urolithin B	100.35	3.47	99.43	> 0.05	101.29	2.51	100.55	> 0.05
6-MF	108.17	5.17	107.15	**< 0.01**	110.94	3.28	111.33	**< 0.01**

	0 min, **AMC**, assay buffer				30 min, **AMC**, assay buffer			
	Mean [%]	S.D. [%]	Median [%]	p	Mean [%]	S.D. [%]	Median [%]	p
Blank	100.00	3.74	100.86	-	100.00	3.04	101.15	-
Quercetin	96.83	2.71	97.68	> 0.05	96.08	2.85	96.23	**< 0.05**
Urolithin A	99.77	3.64	99.64	> 0.05	100.91	2.74	100.85	> 0.05
Urolithin B	99.77	2.01	100.26	> 0.05	99.65	2.55	98.82	> 0.05
6-MF	105.08	1.99	105.23	**< 0.05**	105.44	2.17	105.97	**< 0.01**

Table 1.2.: Descriptive statistical parameters of normalized fluorescent values of blanks, quercetin, urolithin A, urolithin B and 6-MF in DPP IV-containing assay buffer under the tested experimental conditions (n= 8).

	0 min, **no AMC**, DPP IV				30 min, **no AMC**, DPP IV			
	Mean [%]	S.D. [%]	Median [%]	p	Mean [%]	S.D. [%]	Median [%]	p
Blank	100.00	3.20	100.76	-	100.00	3.14	100.59	-
Quercetin	96.76	1.99	97.25	> 0.05	98.12	1.55	98.19	> 0.05
Urolithin A	99.34	2.38	99.35	> 0.05	101.31	1.50	101.65	> 0.05
Urolithin B	99.78	1.68	100.22	> 0.05	101.25	1.41	101.72	> 0.05
6-MF	109.17	5.01	110.04	**< 0.01**	110.45	4.37	112.76	**< 0.01**

	0 min, **AMC**, DPP IV				30 min, **AMC**, DPP IV			
	Mean [%]	S.D. [%]	Median [%]	p	Mean [%]	S.D. [%]	Median [%]	p
Blank	100.00	3.97	100.71	-	100.00	4.65	98.65	-
Quercetin	96.76	2.19	97.17	> 0.05	97.22	2.31	96.63	> 0.05
Urolithin A	102.38	1.83	102.46	> 0.05	101.54	2.83	100.78	> 0.05
Urolithin B	101.46	3.25	101.84	> 0.05	100.92	2.81	100.90	> 0.05
6-MF	107.85	4.93	105.40	**< 0.01**	107.43	4.22	107.74	**< 0.01**

Table 1.3.: Descriptive statistical parameters of normalized fluorescent values of blanks, quercetin, urolithin A, urolithin B and 6-MF in serum under the tested experimental conditions (n= 8).

	0 min, **no AMC**, serum				30 min, **no AMC**, serum			
	Mean [%]	S.D. [%]	Median [%]	p	Mean [%]	S.D. [%]	Median [%]	p
Blank	100.00	3.32	100.28	-	100.00	4.50	101.52	-
Quercetin	93.22	2.16	94.01	**< 0.01**	94.28	1.99	94.22	**< 0.01**
Urolithin A	104.58	1.43	104.34	**< 0.01**	104.43	2.81	104.43	**< 0.05**
Urolithin B	116.70	1.81	116.47	**< 0.01**	112.98	2.97	112.98	**< 0.01**
6-MF	102.43	2.09	102.98	> 0.05	102.61	1.70	102.61	> 0.05

	0 min, **AMC**, serum				30 min, **AMC**, serum			
	Mean [%]	S.D. [%]	Median [%]	p	Mean [%]	S.D. [%]	Median [%]	p
Blank	100.00	4.30	100.01	-	100.00	4.51	100.05	-
Quercetin	92.86	2.03	93.02	**< 0.01**	93.25	2.78	94.61	**< 0.01**
Urolithin A	104.06	3.34	104.56	> 0.05	102.67	1.29	103.01	> 0.05
Urolithin B	113.69	2.38	113.53	**< 0.01**	109.83	2.10	110.08	**< 0.01**
6-MF	101.12	1.53	101.03	> 0.05	101.25	1.34	101.09	> 0.05

E Summary

1 Summary

The growing prevalence of type 2 diabetes mellitus (T2DM) demands novel therapeutic and adjuvant strategies. Polyphenols (PPs) are plant secondary metabolites. Epidemiological studies demonstrate an inverse relationship between their increased intake and the risk of development of T2DM and cardiovascular complications. However, the PPs' mechanism of action remains largely unknown. The present work aimed to expand knowledge regarding the effects of PPs on diabetes relevant molecular targets.

Pycnogenol (PYC) is a standardized pine bark extract which consists of oligomeric and monomeric PPs. Its anti-diabetic effects have been demonstrated in clinical trials. As a part of a human study involving 20 healthy volunteers, the extract's effects on dipeptidyl peptidase IV (DPP IV) were investigated. This protease terminates the insulin secretagogue action of incretins. Its inhibition is a promising strategy in T2DM treatment. This study uncovered that PYC-intake of 100 mg daily over 14 days statistically significantly reduced DPP IV serum concentrations by 8.2 % ($n= 38$, $p= 0.032$). Contrary to expectations, this decrease was not paralleled by a reduction in the serum DPP IV enzymatic activity. To the best of our knowledge, the present study was the first investigating the effects of PPs on DPP IV serum concentrations and activities in humans. The finding that PYC is capable of reducing DPP IV serum concentrations might be important with regard to diabetes, where DPP IV levels are increased.

Screenings for PPs' *in vitro* effects on DPP IV activity were performed employing a purified enzyme. The effects of tested PPs (among which PYC ingredients) at a physiologically relevant concentration of 5 µM were weak (< 10 %) and too small compared to the reference compound sitagliptin, and thus not likely to be clinically relevant. This result is in discordance with some published data, but consistent with the outcome from the present human study. In addition, fluorescence interactions with the experimental setup were registered: under certain conditions urolithin B exhibited an autofluorescence which might mask eventual inhibitory activity. Quercetin quenched the fluorescence slightly which might contribute to false positive results. No statistically significant effects of selected constituents and metabolites of PYC on the total DPP IV protein expression were observed in 3T3-L1 adipocytes. Thus, the lower DPP IV *in vivo* concentrations after intake of PYC cannot be explained with down-regulation of the DPP IV expression in adipocytes.

Akt kinase is responsible for the transmission of insulin signals and its dysregulation is related to insulin resistance and plays an important role in development of cardiovascular complications in T2DM. Thus, the modulation of the phosphorylation status of endothelial Akt-kinase, respectively its activity, might be a promising strategy in the management of these pathologies. This work aimed to uncover the effects of PPs from different structural subclasses on Akt-phosphorylation (pAkt) in endothelial cells (Ea.hy926). Short-term effects (5 – 30 min) were investigated at a concentration of 10 µM. In a pilot study two model PPs induced a moderate, but reproducible inhibition of pAkt Ser473 of 52.37 ± 21.01 % (quercetin; p= 0.006, n= 3) and 37.79 ± 7.14 % (resveratrol; p= 0.021, n= 4) compared to the negative control. A primary screening with Western blot analysis investigated the effects of eight compounds from different subclasses on pAkt Ser473 and Thr308 to reveal whether the observed inhibition PPs a group effect or specific to certain compounds. In addition to resveratrol and quercetin, statistically significant inhibitions of pAkt Ser473 were induced by luteolin (29.96 ± 11.06 %, p< 0.01, n= 6) and apigenin (22.57 ± 10.30 %, p< 0.01, n= 6). In contrast, genistein, 3,4,5-trimethoxystilbene, taxifolin and (+)-catechin caused no inhibition. A strong positive and statistically significant correlation between the mean inhibitory effects of the tested PPs on both Akt-residues Ser473 and Thr308 (r= 0.9478, p= 0.0003) was determined. A comprehensive secondary screening via ELISA involving 44 compounds from nine structural groups quantified the effects of PPs on pAkt Ser473 to uncover potential structure-activity features. The most potent inhibitors were luteolin (44.31 ± 17.95 %), quercetin (35.71 ± 8.33 %), urolithin A (35.28 ± 11.80 %), apigenin (31.79 ± 6.16 %), fisetin (28.09 ± 9.09 %), and resveratrol (26.04 ± 5.58 %). These effects were statistically significant (p< 0.01, n= 3 to 6). Further lead structure optimization might be based on the fact that the effects of luteolin and resveratrol also differed statistically significantly from each other (p= 0.008).

To the best of our knowledge, the present study is the first to compare quantitatively the short term effects of PPs from different subclasses on pAkt in endothelial cells. Basic structure-activity relationships revealed that for flavones and flavonols the presence of a C2=C3 double bound (ring C) was essential for inhibitory activity and hydroxylation on the *m*- and *p*- positions in the ring B contributed to it. For stilbenoids, three free OH-groups appeared to be optimal. The comparison of the inhibitory potentials of ellagic acid and its microbial metabolites showed that urolithin A was statistically significantly more effective than its progenitor compound. Despite their structural similarities, the only active compound among all urolithins tested was urolithin A, hydroxylated at the C3 and C8 positions. This suggested a specific effect for urolithin A. Based on the common structural determinants and molecular geometry of the most active PPs a pharmacophore model regarding Akt-inhibition was proposed.

In summary, the effects of a wide variety of PPs from diverse structural subclasses on the *in vitro* phosphorylation of endothelial Akt were quantitatively analyzed for the first time, the effects of previously undescribed compounds were determined and structure activity

relationships were elucidated. The inhibitory potential of individual PPs might be beneficial in cases of sustained over-activation of Akt-kinase and its substrates such as S6 kinase as reported for certain T2DM-related pathological states, such as insulin resistance, endothelial dysfunction, excessive angiogenesis, vascular calcification, and insulin triggered DNA-damage. The results of the present work suggest potential molecular mechanisms of action of PP involving Akt-inhibition and DPP IV-down-regulation and thus contribute to the understanding of anti-diabetic effects of these compounds on the molecular level.

2 Zusammenfassung

Diabetes mellitus Typ 2 (DMT2) und seine Spätkomplikationen sind ein maßgeblicher Grund für Morbidität und Mortalität. Die steigende Prävalenz der Krankheit erfordert die Entwicklung neuer therapeutischer und prophylaktischer Strategien. Publizierte Daten deuten darauf hin, dass diätetische Polyphenole (PP) sowohl zur Prävention des Diabetes beitragen als auch therapiebegleitend sinnvoll eingesetzt werden können. Allerdings sind ihre Wirkmechanismen nicht vollständig aufgeklärt. Das Ziel der Arbeit war die Charakterisierung zellulärer Wirkungen von PP, die eine Relevanz in der unterstützenden Behandlung von DMT2 und denen Spätkomplikationen haben könnten.

Pycnogenol® (PYC) ist ein standardisierter Kiefernrindenextrakt, der oligomere und monomere PP enthält. PYC hat in klinischen Studien mit Diabetikern viele vorteilhafte anti-diabetische und protektive Effekte gezeigt. Die Wirkungen des Extraktes auf die Serumkonzentration und die enzymatische Aktivität der Dipeptidylpeptidase IV (DPP IV) wurden im Rahmen einer klinischen Studie mit 20 gesunden Probanden untersucht. DPP IV ist eine Serin-Exopeptidase, die Inkretinhormone spaltet und damit deren Wirkung auf die Insulin-Freisetzung beendet. Nach PYC-Einnahme zeigte sich im Vergleich zu dem Kontrollzustand eine statistisch signifikante ($p= 0{,}032$, $n= 38$) Abnahme der DPP-IV-Konzentration von 8.2 %. Allerdings wurden keine deutlichen Änderungen der mittleren DPP-IV-Aktivität durch die PYC-Behandlung hervorgerufen. Nach unserem Wissensstand war die klinische Studie die erste ihrer Art, welche die Effekte von PP auf die Serumkonzentration und die enzymatischen Aktivität von DPP IV im Menschen untersuchte. Da die Serumspiegel von DPP IV bei diabetischen Patienten im Vergleich zu gesunden Menschen erhöht sind, könnte der beobachtete Effekt vorteilhaft sein.

Als Nächstes sollten die Effekte von einzelnen PP und ihren Metaboliten auf die enzymatische Aktivität der DPP IV *in vitro* untersucht werden. Die Ergebnisse zeigten nur eine geringe Hemmung (< 10 %) der DPP IV Aktivität durch die PP (auch Inhaltsstoffe von PYC und deren Metaboliten) in einer Konzentration von 5 µM, verglichen zur initialen 100 %-igen Enzymaktivität. Die Positivkontrolle (50 nM Sitagliptin) verursachte hingegen eine starke Senkung der Aktivität von DPP IV. Es ist daher unwahrscheinlich, dass die Effekte der PP eine klinische Relevanz haben. Die Ergebnisse stehen im Widerspruch zu bisher veröffentlichten Daten, unterstützen aber die Resultate aus der vorliegenden Humanstudie. Zusätzlich durchgeführte Fluoreszenzmessungen deuten darauf hin, dass Quercetin und Urolithin B unter definierten Bedingungen in der Lage sind, die Ergebnisse des DPP IV-Inhibitoren-Screening-Assays unspezifisch zu beeinflussen. *In-vitro*-Experimente in 3T3-L1 differenzierten Adipozyten zeigten zudem keine statistisch signifikanten Effekte auf die Proteinexpression von DPP IV – weder für einzelne

Bestandteile noch für Metabolite von PYC. Somit konnte die beobachtete Abnahme der DPP-IV-Konzentrationen *in vivo* nach der Einnahme von PYC nicht durch eine Herunterregulierung der DPP-IV-Expression in Adipozyten erklärt werden.

Die Akt-Kinase/PKB (Protein Kinase B) spielt eine wesentliche Rolle bei der Vermittlung der Effekte des Insulins auf intrazellulärer Ebene bzw. bei der Pathophysiologie von DMT2 sowie dessen vaskulären Spätfolgen. Die Modulation von Akt in Endothelzellen ist ein vielversprechender Ansatz, um pathophysiologische Veränderungen zu beeinflussen, die für diabetische kardiovaskuläre Spätkomplikationen verantwortlich sind. Daher sollte in der vorliegenden Arbeit der Einfluss von PP aus verschiedenen strukturellen Subklassen auf die Akt-Phosphorylierung (pAkt) an Ser 473 und Thr 308 in Endothelzellen (EA.hy926) *in vitro* untersucht werden (10 µM, 5 – 30 min). In der Pilotstudie hemmten beide Modellverbindungen die Akt-Phosphorylierung (pAkt Ser473) statistisch signifikant mit 52,37 ± 21,01 % (Quercetin; p= 0,006, n= 3) und 37,79 ± 7,14 % (Resveratrol; p= 0,021, n= 4). Im Primärscreening wurden acht Verbindungen verschiedener Subklassen einbezogen. Die Substanzen, welche neben Quercetin und Resveratrol die stärksten Hemmungsaktivitäten auf pAkt Ser473 (Mittelwert ± Standardabweichung) hatten, waren Luteolin (29,96 ± 11,06 %, p< 0,01, n= 6) und Apigenin (22,57 ± 10,30 %, p< 0,01, n= 6). Im Gegensatz dazu zeigten Genistein, 3,4,5-Trimethoxy-trans-stilben, Taxifolin und (+)-Catechin keine Hemmung. Die inhibierenden Effekte der PP auf pAkt Thr308 und pAkt Ser473 korrelierten positiv und statistisch signifikant miteinander (r= 0,9478, p= 0,0003). In einem sekundären Screening sollten die Effekte diverser PP auf die Phosphorylierung von Akt an Ser 473 in Endothelzellen mittels quantitativen ELISA detailliert untersucht werden. Die Verbindungen mit den größten Hemmungseffekte waren Luteolin (44,31 ± 17,95 %), Quercetin (35,71 ± 8,33 %), Urolithin A (35,28 ± 11,80 %), Apigenin (31,79 ± 6,16 %), Fisetin (28,09 ± 9,09 %), und Resveratrol (26,04 ± 5,58 %). Diese Hemmungen waren statistisch signifikant (p< 0,01, n= 3 - 6). Zusätzlich war die Wirkung von Luteolin und Resveratrol statistisch signifikant verschieden (p= 0,008), was einer weiteren Leitstrukturoptimierung dienen kann.

Der quantitative Vergleich der Substanzaktivitäten war die Grundlage für nachfolgende Struktur-Wirkungsuntersuchungen (SAR). Die Doppelbindung C2=C3 (Ring C) war für die Inhibitionseffekte bei Flavonen und Flavon-3-olen von wesentlicher Bedeutung. Die Anwesenheit einer *meta*- und einer *para*- OH-Gruppe (Ring B) trug sehr wahrscheinlich zu den Hemmeffekten bei. Bei den Stilbenoiden hingegen waren drei freie OH-Gruppen (bei Resveratrol) optimal für die Aktivität. Der mikrobielle Metabolit der Ellagsäure, Urolithin A, hemmte im Vergleich zur Muttersubstanz die Akt-Phosphorylierung statistisch signifikant. Im Gegensatz dazu zeigten die anderen Urolithine trotz der strukturellen Ähnlichkeit nur geringe Effekte. Dies deutet auf einen spezifischen Effekt von Urolithin A hin. Basierend auf den gemeinsamen Merkmalen, die für die Aktivität wichtig sein können

und der molekularen Geometrie der aktivsten PP, wurde ein Pharmakophormodell für die Akt-Hemmung vorgeschlagen.

Zusammengefasst ist die vorliegende Studie nach unserem Wissensstand die erste, welche die Auswirkungen einer Vielzahl von PP verschiedener struktureller Subklassen auf die Akt-Phosphorylierung in Endothelzellen quantitativ verglich. Es wurden Effekte von zuvor nicht beschriebenen Verbindungen und Struktur-Aktivitäts-Beziehungen ermittelt. Die hemmenden Eigenschaften einzelner PP auf die Akt-Kinase könnten im Fall einer anhaltenden Überaktivierung der Akt-Kinase und ihren Substraten wie die S6-Kinase vorteilhaft sein. Dieser Zustand wurde bei bestimmten DMT2-verwandten pathologischen Prozessen, wie Insulinresistenz, endotheliale Dysfunktion, übermäßige Angiogenese, vaskuläre Calcifizierung und Insulin-induzierte-DNA-Schäden beobachtet. Die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass die Wirkmechanismen von PP die Akt-Inhibierung und DPP IV-Herunterregulierung umfassen könnten, was zum Verständnis der anti-diabetischen und protektiven Wirkungen dieser Verbindungen auf molekularer Ebene beitragen kann.

F Abbreviations in alphabetical order

3-MS - 3,4',5-trimethoxy-trans-stilbene

6-MF - 6-methoxyflavone

ADAM - A disintegrin and metalloproteinase

ADCP2 - Adenosine deaminase complexing protein 2 (see DPP IV)

ADN - Adiponectin

AGE - Advanced glycation end products

AMC - 7-aminomethyl-4-methylcoumarin

AMP - Adenosine monophosphate

AMPK - 5'adenosine monophosphate-activated protein kinase

ANOVA - Analysis of variance

Aro|Hyd 1 - Aromatic or hydrophobic regions

AS160 - 160 kDa Akt substrate

ATP - Adenosine triphosphate

ATRA - All-trans-retinoic acid

BCA - Bicinchoninic acid

BSA - Bovine serum albumin

CaMKKβ - Ca2+/calmodulin-dependent protein kinase kinase β

CAT - central catalytic kinase domain

CD26 - Cluster of differentiation 26 (see DPP IV)

COX - Cyclooxigenase

CVD - Cardiovascular diseases

DASH - DPP IV activity and/or structure homologs

DMEM - Dulbecco's modified essential medium

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

Don & Acc - Hydrogen bond donor & hydrogen bond acceptor

DPP IV - Dipeptidyl peptidase IV

DTT - Dithiothreitol

EC - Endothelial cells

ED - Endothelial dysfunction

EGCG - Epigallocatechin gallate

EGF - Epidermal growth factor

ELISA - Enzyme-linked immunosorbent assay

eNOS, NOSIII - Endothelial nitric oxide synthase

ERK - Extracellular signal-regulated kinases

ET-1 - Endothelin-1

EXT - C-terminal extension

FBS - Fetal bovine serum

FLU - Fluorescence

FoxO - Forkhead box O family transcription factors

GLP-1 - Glucagon-like peptide-1

GLUT4 - Glucose transporter 4

Gly - Glycine

GPCR - G-protein coupled receptor

GSK-3β - Glycogen synthase kinase-3β

HbA1c - Hemoglobin A1C (Glycated Hemoglobin)

HDL - High density lipoprotein

HG - High glucose - 25.0 mM glucose

HNF-1α - Hepatocyte nuclear factor-1α

HOMA - Homeostatic model assessment of insulin resistance

HRP - Horseradish peroxidase

HSD - Honestly significant difference

HUVEC - Human umbilical vein endothelial cells

IBMX - 3-isobutyl-1-methylxanthine

IGT - Impaired glucose tolerance

IGF-1 - Insulin-like growth factor-1

IFG - Impaired fasting glucose

iNOS, NOS II - Inducible nitric oxide synthase

IL-6 - Interleukin-6

IRS - Insulin receptor substrate

JNK - c-Jun N-terminal kinase

LADA - Latent autoimmune diabetes of adults

LG - Low glucose - 5.5 mM glucose

LINK - Linker region

LKB-1 - Liver kinase B1

MAPK - Mitogen-activated protein kinase

MMP - Matrix metalloproteinases

MOE - Molecular Operating Environment

MODY - Maturity onset diabetes of the young

mTORC1- Mechanistic target of rapamycin complex 1

mTORC2 - Mechanistic target of rapamycin complex 2

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NO - Nitric oxide

Nrf-2 - Nuclear transcription factor erythroid-2 related factor-2

N.S. - Non statistically significant

O.D. - Optical density

OGTT - Oral glucose tolerance test

OGTT-1(2)-G – OGTT performed with glucose

OGTT-1(2)-R – OGTT performed with rice

pAkt - Akt-phosphorylation

PCR - Polymerase chain reaction

PDGF - platelet derived growth factor

PDK1 - Phosphoinositide-dependent kinase 1

PH - pleckstrin homology domain

PI - Protease Inhibitor cocktail

PI3K - Phosphoinositide-3-kinase

PiN - Planar projection of an aromatic ring

PIP2 - Phosphatidylinositol-(4,5)-bisphosphate

PIP3 - Phosphatidylinositol-(3,4,5)-trisphosphate

$pK_{a/b}$-value - Logarithmic acid/base dissociation constant

PKB - Protein kinase B

PKC - Protein kinase C

PMA - Phorbol ester

PPs - Polyphenols

PPAR-γ - Peroxisome proliferator-activated receptor-γ

Pro - Proline

PTEN - Phosphatase and tensin homolog

PYC - Pycnogenol®

QC - Quality controls

qp - Quality of prediction

Que - Quercetin

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RSV - Resveratrol

RTK - Receptor tyrosine kinases

S6K1 - Ribosomal protein S6 kinase beta-1

SAR - Structure-activity relationship studies

S.D. - Standard deviation

SDS - Sodium dodecyl sulfate

Ser - Serine

T1DM - Type 1 diabetes mellitus

T2DM (DMT2) - Type 2 diabetes mellitus (Diabetes mellitus Typ 2)

TEMED - Tetramethylethylenediamine

Thr - Threonine

TNF-α - Tumor necrosis factor-α

TRIS - Tris(hydroxymethyl)aminomethane

TSC-2 - Tuberous sclerosis complex 2

VEGF - Vascular endothelial growth factor

WHO - World Health Organization

G References

1. *Global report on diabetes.* 2016, World Health Organization (WHO): http://www.who.int. p. 6; p. 83.
2. American Diabetes Association. *Statistics About Diabetes*. 2018 Last Update: 22.03.2018, [cited 2018 03.12.]; Available from: http://www.diabetes.org/diabetes-basics/statistics/.
3. Eurostat. *Chronic diabetes affects millions of people in the EU*. 2017 Last Update: 13.11.2017, [cited 2018 03.12.]; Available from: http://www.ec.europa.eu/eurostat/web/products-eurostat-news/-/EDN-20171113-1?inheritRedirect=true.
4. Ahlqvist, E., Storm, P., Käräjämäki, A., Martinell, M., Dorkhan, M., Carlsson, A., Vikman, P., Prasad, R.B., Aly, D.M., Almgren, P., Wessman, Y., Shaat, N., Spégel, P., Mulder, H., Lindholm, E., Melander, O., Hansson, O., Malmqvist, U., Lernmark, Å., Lahti, K., Forsén, T., Tuomi, T., Rosengren, A.H., Groop, L., *Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables.* Lancet Diabetes Endocrinol, 2018. **6**(5): 361-369.
5. Ahmed, R.G., *Ahmed, R.G., 2018. Editor for "Diabetes and its complications book" on InTech - open science, ISBN: 978-1-78923-035-2 and Print ISBN: 978-1-78923-034-5. https://www.intechopen.com/books/diabetes-and-its-complications*. 2018.
6. *7. Diabetes Technology: Standards of Medical Care in Diabetes - 2019.* Diabetes Care, 2019. **42**(Supplement 1): S71.
7. *2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes - 2019.* Diabetes Care, 2019. **42**(Supplement 1): S13-S28.
8. Huang, Y., Cai, X., Mai, W., Li, M., Hu, Y., *Association between prediabetes and risk of cardiovascular disease and all cause mortality: systematic review and meta-analysis.* BMJ, 2016. **355**.
9. Yaribeygi, H., Farrokhi, F.R., Butler, A.E., Sahebkar, A., *Insulin resistance: Review of the underlying molecular mechanisms.* J Cell Physiol, 2018. **234**(6): 8152-8161.
10. Gayoso-Diz, P., Otero-González, A., Rodriguez-Alvarez, M.X., Gude, F., García, F., De Francisco, A., Quintela, A.G., *Insulin resistance (HOMA-IR) cut-off values and the metabolic syndrome in a general adult population: effect of gender and age: EPIRCE cross-sectional study.* BMC Endocr Disord, 2013. **13**(1): 47.
11. Janus, A., Szahidewicz-Krupska, E., Mazur, G., Doroszko, A., *Insulin Resistance and Endothelial Dysfunction Constitute a Common Therapeutic Target in Cardiometabolic Disorders.* Mediators Inflamm, 2016. **2016**: 10.
12. Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M.E., Pratipanawatr, T., DeFronzo, R.A., Kahn, C.R., Mandarino, L.J., *Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle.* J Clin Invest, 2000. **105**(3): 311-320.
13. Eringa, E.C., Serne, E.H., Meijer, R.I., Schalkwijk, C.G., Houben, A.J., Stehouwer, C.D., Smulders, Y.M., van Hinsbergh, V.W., *Endothelial dysfunction in (pre)diabetes: characteristics, causative mechanisms and pathogenic role in type 2 diabetes.* Rev Endocr Metab Disord, 2013. **14**(1): 39-48.
14. *Global Guidelines for managing older people with type 2 diabetes* 2015, International Diabetes Federation: http://www.idf.org. p. 35.
15. Lean, M.E., Leslie, W.S., Barnes, A.C., Brosnahan, N., Thom, G., McCombie, L., Peters, C., Zhyzhneuskaya, S., Al-Mrabeh, A., Hollingsworth, K.G., Rodrigues, A.M., Rehackova, L., Adamson, A.J., Sniehotta, F.F., Mathers, J.C., Ross, H.M., McIlvenna, Y., Stefanetti, R., Trenell, M., Welsh, P., Kean, S., Ford, I., McConnachie, A., Sattar, N., Taylor, R., *Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial.* Lancet, 2018. **391**(10120): 541-551.
16. Esposito, K., Maiorino, M.I., Petrizzo, M., Bellastella, G., Giugliano, D., *The effects of a Mediterranean diet on the need for diabetes drugs and remission of newly diagnosed type 2 diabetes: follow-up of a randomized trial.* Diabetes Care, 2014. **37**(7): 1824-30.
17. Marín-Peñalver, J.J., Martín-Timón, I., Sevillano-Collantes, C., Del Cañizo-Gómez, F.J., *Update on the treatment of type 2 diabetes mellitus.* World J Diabetes, 2016. **7**(17): 354-395.
18. Othman, E.M., Oli, R.G., Arias-Loza, P.A., Kreissl, M.C., Stopper, H., *Metformin Protects Kidney Cells From Insulin-Mediated Genotoxicity In Vitro and in Male Zucker Diabetic Fatty Rats.* Endocrinology, 2016. **157**(2): 548-59.
19. Meng, S., Cao, J., He, Q., Xiong, L., Chang, E., Radovick, S., Wondisford, F.E., He, L., *Metformin Activates AMP-activated Protein Kinase by Promoting Formation of the αβγ Heterotrimeric Complex.* J Biol Chem, 2015. **290**(6): 3793-3802.

20. Mackenzie, R.W.A., Elliott, B.T., *Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes.* Diabetes Metab Syndr Obes, 2014. **7**: 55-64.
21. Valentine, R.J., Coughlan, K.A., Ruderman, N.B., Saha, A.K., *Insulin inhibits AMPK activity and phosphorylates AMPK Ser(4)(8)(5)/(4)(9)(1) through Akt in hepatocytes, myotubes and incubated rat skeletal muscle.* Arch Biochem Biophys, 2014. **562**: 62-9.
22. Morrison, A., Yan, X., Tong, C., Li, J., *Acute rosiglitazone treatment is cardioprotective against ischemia-reperfusion injury by modulating AMPK, Akt, and JNK signaling in nondiabetic mice.* Am J Physiol Heart Circ Physiol, 2011. **301**(3): H895-902.
23. Wei, R., Ma, S., Wang, C., Ke, J., Yang, J., Li, W., Liu, Y., Hou, W., Feng, X., Wang, G., Hong, T., *Exenatide exerts direct protective effects on endothelial cells through the AMPK/Akt/eNOS pathway in a GLP-1 receptor-dependent manner.* Am J Physiol Endocrinol Metab, 2016. **310**(11): E947-57.
24. Gallego-Colon, E., Klych-Ratuszny, A., Kosowska, A., Garczorz, W., Aghdam, M.R.F., Wozniak, M., Francuz, T., *Exenatide modulates metalloproteinase expression in human cardiac smooth muscle cells via the inhibition of Akt signaling pathway.* Pharmacol Rep, 2018. **70**(1): 178-183.
25. Al-Damry, N.T., Attia, H.A., Al-Rasheed, N.M., Al-Rasheed, N.M., Mohamad, R.A., Al-Amin, M.A., Dizmiri, N., Atteya, M., *Sitagliptin attenuates myocardial apoptosis via activating LKB-1/AMPK/Akt pathway and suppressing the activity of GSK-3beta and p38alpha/MAPK in a rat model of diabetic cardiomyopathy.* Biomed Pharmacother, 2018. **107**: 347-358.
26. Kaji, K., Nishimura, N., Seki, K., Sato, S., Saikawa, S., Nakanishi, K., Furukawa, M., Kawaratani, H., Kitade, M., Moriya, K., Namisaki, T., Yoshiji, H., *Sodium glucose cotransporter 2 inhibitor canagliflozin attenuates liver cancer cell growth and angiogenic activity by inhibiting glucose uptake.* Int J Cancer, 2018. **142**(8): 1712-1722.
27. Hawley, S.A., Ford, R.J., Smith, B.K., Gowans, G.J., Mancini, S.J., Pitt, R.D., Day, E.A., Salt, I.P., Steinberg, G.R., Hardie, D.G., *The Na+/Glucose Cotransporter Inhibitor Canagliflozin Activates AMPK by Inhibiting Mitochondrial Function and Increasing Cellular AMP Levels.* Diabetes, 2016. **65**(9): 2784-2794.
28. Manning, B.D., Toker, A., *AKT/PKB Signaling: Navigating the Network.* Cell, 2017. **169**(3): 381-405.
29. Farese, R.V., Sajan, M.P., Standaert, M.L., *Insulin-sensitive protein kinases (atypical protein kinase C and protein kinase B/Akt): actions and defects in obesity and type II diabetes.* Exp Biol Med (Maywood), 2005. **230**(9): 593-605.
30. Kumar, C.C., Madison, V., *AKT crystal structure and AKT-specific inhibitors.* Oncogene, 2005. **24**(50): 7493-501.
31. Peng, X.D., Xu, P.Z., Chen, M.L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararajan, D., Chen, W.S., Crawford, S.E., Coleman, K.G., Hay, N., *Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2.* Genes Dev, 2003. **17**(11): 1352-65.
32. George, S., Rochford, J.J., Wolfrum, C., Gray, S.L., Schinner, S., Wilson, J.C., Soos, M.A., Murgatroyd, P.R., Williams, R.M., Acerini, C.L., Dunger, D.B., Barford, D., Umpleby, A.M., Wareham, N.J., Davies, H.A., Schafer, A.J., Stoffel, M., O'Rahilly, S., Barroso, I., *A Family with Severe Insulin Resistance and Diabetes Mellitus due to a Missense Mutation in AKT2.* Science, 2004. **304**(5675): 1325-1328.
33. Scheid, M.P., Marignani, P.A., Woodgett, J.R., *Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B.* Mol Cell Biol, 2002. **22**(17): 6247-60.
34. Franke, T.F., Hornik, C.P., Segev, L., Shostak, G.A., Sugimoto, C., *PI3K/Akt and apoptosis: size matters.* Oncogene, 2003. **22**(56): 8983-98.
35. Calleja, V., Laguerre, M., Parker, P.J., Larijani, B., *Role of a novel PH-kinase domain interface in PKB/Akt regulation: structural mechanism for allosteric inhibition.* PLoS Biol, 2009. **7**(1): e17.
36. Huang, J., Dibble, C.C., Matsuzaki, M., Manning, B.D., *The TSC1-TSC2 Complex Is Required for Proper Activation of mTOR Complex 2.* Mol Cell Biol, 2008. **28**(12): 4104-4115.
37. Ebner, M., Sinkovics, B., Szczygiel, M., Ribeiro, D.W., Yudushkin, I., *Localization of mTORC2 activity inside cells.* J Cell Biol, 2017. **216**(2): 343-353.
38. Mahajan, K., Coppola, D., Challa, S., Fang, B., Chen, Y.A., Zhu, W., Lopez, A.S., Koomen, J., Engelman, R.W., Rivera, C., Muraoka-Cook, R.S., Cheng, J.Q., Schönbrunn, E., Sebti, S.M., Earp, H.S., Mahajan, N.P., *Ack1 Mediated AKT/PKB Tyrosine 176 Phosphorylation Regulates Its Activation.* PLoS One, 2010. **5**(3): e9646.
39. Zheng, Y., Peng, M., Wang, Z., Asara, J.M., Tyner, A.L., *Protein Tyrosine Kinase 6 Directly Phosphorylates AKT and Promotes AKT Activation in Response to Epidermal Growth Factor.* Mol Cell Biol, 2010. **30**(17): 4280-4292.
40. Liu, P., Begley, M., Michowski, W., Inuzuka, H., Ginzberg, M., Gao, D., Tsou, P., Gan, W., Papa, A., Kim, B.M., Wan, L., Singh, A., Zhai, B., Yuan, M., Wang, Z., Gygi, S.P., Lee, T.H., Lu, K.P., Toker, A., Pandolfi, P.P., Asara, J.M., Kirschner, M.W., Sicinski, P., Cantley, L., Wei, W., *Cell-*

cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. Nature, 2014. **508**(7497): 541-5.
41. Guo, H., Gao, M., Lu, Y., Liang, J., Lorenzi, P.L., Bai, S., Hawke, D.H., Li, J., Dogruluk, T., Scott, K.L., Jonasch, E., Mills, G.B., Ding, Z., *Coordinate phosphorylation of multiple residues on single AKT1 and AKT2 molecules.* Oncogene, 2014. **33**(26): 3463-72.
42. Leslie, N.R., Longy, M., *Inherited PTEN mutations and the prediction of phenotype.* Semin Cell Dev Biol, 2016. **52**: 30-38.
43. Kuo, Y.C., Huang, K.Y., Yang, C.H., Yang, Y.S., Lee, W.Y., Chiang, C.W., *Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt.* J Biol Chem, 2008. **283**(4): 1882-92.
44. Zhang, J., Gao, Z., Yin, J., Quon, M.J., Ye, J., *S6K Directly Phosphorylates IRS-1 on Ser-270 to Promote Insulin Resistance in Response to TNF-α Signaling through IKK2.* J Biol Chem, 2008. **283**(51): 35375-35382.
45. Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J., Thomas, G., *Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity.* Nature, 2004. **431**(7005): 200-5.
46. Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P.K., Baselga, J., Rosen, N., *AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity.* Cancer Cell, 2011. **19**(1): 58-71.
47. King, T.D., Song, L., Jope, R.S., *AMP-activated protein kinase (AMPK) activating agents cause dephosphorylation of Akt and glycogen synthase kinase-3.* Biochem Pharmacol, 2006. **71**(11): 1637-47.
48. Hawley, S.A., Ross, F.A., Gowans, G.J., Tibarewal, P., Leslie, N.R., Hardie, D.G., *Phosphorylation by Akt within the ST loop of AMPK-alpha1 down-regulates its activation in tumour cells.* Biochem J, 2014. **459**(2): 275-87.
49. Mendoza, M.C., Er, E.E., Blenis, J., *The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and Compensation.* Trends Biochem Sci, 2011. **36**(6): 320-328.
50. Gonzalez, E., McGraw, T.E., *Insulin-modulated Akt subcellular localization determines Akt isoform-specific signaling.* Proc Natl Acad Sci U S A, 2009. **106**(17): 7004-9.
51. Chakraborty, A., Koldobskiy, M.A., Bello, N.T., Maxwell, M., Potter, J.J., Juluri, K.R., Maag, D., Kim, S., Huang, A.S., Dailey, M.J., Saleh, M., Snowman, A.M., Moran, T.H., Mezey, E., Snyder, S.H., *Inositol pyrophosphates inhibit Akt signaling, thereby regulating insulin sensitivity and weight gain.* Cell, 2010. **143**(6): 897-910.
52. Peck, G.R., Chavez, J.A., Roach, W.G., Budnik, B.A., Lane, W.S., Karlsson, H.K.R., Zierath, J.R., Lienhard, G.E., *Insulin-stimulated Phosphorylation of the Rab GTPase-activating Protein TBC1D1 Regulates GLUT4 Translocation.* J Biol Chem, 2009. **284**(44): 30016-30023.
53. Jope, R.S., Johnson, G.V.W., *The glamour and gloom of glycogen synthase kinase-3.* Trends Biochem Sci, 2004. **29**(2): 95-102.
54. Meijer, L., Flajolet, M., Greengard, P., *Pharmacological inhibitors of glycogen synthase kinase 3.* Trends Pharmacol Sci, 2004. **25**(9): 471-480.
55. Saxton, R.A., Sabatini, D.M., *mTOR Signaling in Growth, Metabolism, and Disease.* Cell, 2017. **168**(6): 960-976.
56. Zoncu, R., Sabatini, D.M., Efeyan, A., *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nat Rev Mol Cell Biol, 2011. **12**(1): 21-35.
57. Webb, A.E., Brunet, A., *FOXO transcription factors: key regulators of cellular quality control.* Trends Biochem Sci, 2014. **39**(4): 159-69.
58. Lu, M., Wan, M., Leavens, K.F., Chu, Q., Monks, B.R., Fernandez, S., Ahima, R.S., Ueki, K., Kahn, C.R., Birnbaum, M.J., *Insulin regulates liver metabolism in vivo in the absence of hepatic Akt and Foxo1*. Nat Med, 2012. **18**(3): 388-95.
59. Abeyrathna, P., Su, Y., *The critical role of Akt in cardiovascular function.* Vascul Pharmacol, 2015. **74**: 38-48.
60. Ruan, G.-X., Kazlauskas, A., *VEGF-A engages at least three tyrosine kinases to activate PI3K/Akt.* Cell Cycle, 2012. **11**(11): 2047-2048.
61. Förstermann, U., Sessa, W.C., *Nitric oxide synthases: regulation and function.* Eur Heart J, 2012. **33**(7): 829-837.
62. Federici, M., Menghini, R., Mauriello, A., Hribal, M.L., Ferrelli, F., Lauro, D., Sbraccia, P., Spagnoli, L.G., Sesti, G., Lauro, R., *Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells.* Circulation, 2002. **106**(4): 466-72.
63. Yu, H., Littlewood, T., Bennett, M., *Akt isoforms in vascular disease.* Vascul Pharmacol, 2015. **71**: 57-64.

64. Brown, J.S., Banerji, U., *Maximising the potential of AKT inhibitors as anti-cancer treatments.* Pharmacol Ther, 2017. **172**: 101-115.
65. Ruderman, N.B., Carling, D., Prentki, M., Cacicedo, J.M., *AMPK, insulin resistance, and the metabolic syndrome.* J Clin Invest, 2013. **123**(7): 2764-2772.
66. Coughlan, K.A., Valentine, R.J., Ruderman, N.B., Saha, A.K., *AMPK activation: a therapeutic target for type 2 diabetes?* Diabetes Metab Syndr Obes, 2014. **7**: 241-253.
67. Oakhill, J.S., Scott, J.W., Kemp, B.E., *AMPK functions as an adenylate charge-regulated protein kinase.* Trends Endocrinol Metab, 2012. **23**(3): 125-32.
68. Stahmann, N., Woods, A., Carling, D., Heller, R., *Thrombin Activates AMP-Activated Protein Kinase in Endothelial Cells via a Pathway Involving Ca(2+)/Calmodulin-Dependent Protein Kinase Kinase β.* Mol Cell Biol, 2006. **26**(16): 5933-5945.
69. Garcia-Haro, L., Garcia-Gimeno, M.A., Neumann, D., Beullens, M., Bollen, M., Sanz, P., *Glucose-dependent regulation of AMP-activated protein kinase in MIN6 beta cells is not affected by the protein kinase A pathway.* FEBS Lett, 2012. **586**(23): 4241-4247.
70. Zhang, Y., Lee, T.S., Kolb, E.M., Sun, K., Lu, X., Sladek, F.M., Kassab, G.S., Garland, T., Jr., Shyy, J.Y., *AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress.* Arterioscler Thromb Vasc Biol, 2006. **26**(6): 1281-7.
71. Price, N.L., Gomes, A.P., Ling, A.J.Y., Duarte, F.V., Martin-Montalvo, A., North, B.J., Agarwal, B., Ye, L., Ramadori, G., Teodoro, J.S., Hubbard, B.P., Varela, A.T., Davis, J.G., Varamini, B., Hafner, A., Moaddel, R., Rolo, A.P., Coppari, R., Palmeira, C.M., de Cabo, R., Baur, J.A., Sinclair, D.A., *SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function.* Cell Metab, 2012. **15**(5): 675-690.
72. Lee, Y.S., Kim, W.S., Kim, K.H., Yoon, M.J., Cho, H.J., Shen, Y., Ye, J.-M., Lee, C.H., Oh, W.K., Kim, C.T., Hohnen-Behrens, C., Gosby, A., Kraegen, E.W., James, D.E., Kim, J.B., *Berberine, a Natural Plant Product, Activates AMP-Activated Protein Kinase With Beneficial Metabolic Effects in Diabetic and Insulin-Resistant States.* Diabetes, 2006. **55**(8): 2256.
73. Lee, W.J., Song, K.H., Koh, E.H., Won, J.C., Kim, H.S., Park, H.S., Kim, M.S., Kim, S.W., Lee, K.U., Park, J.Y., *Alpha-lipoic acid increases insulin sensitivity by activating AMPK in skeletal muscle.* Biochem Biophys Res Commun, 2005. **332**(3): 885-91.
74. Desjardins, E.M., Steinberg, G.R., *Emerging Role of AMPK in Brown and Beige Adipose Tissue (BAT): Implications for Obesity, Insulin Resistance, and Type 2 Diabetes.* Curr Diab Rep, 2018. **18**(10): 80.
75. Greig, F.H., Ewart, M.-A., McNaughton, E., Cooney, J., Spickett, C.M., Kennedy, S., *The hypotensive effect of acute and chronic AMP-activated protein kinase activation in normal and hyperlipidemic mice.* Vascul Pharmacol, 2015. **74**: 93-102.
76. Galic, S., Loh, K., Murray-Segal, L., Steinberg, G.R., Andrews, Z.B., Kemp, B.E., *AMPK signaling to acetyl-CoA carboxylase is required for fasting- and cold-induced appetite but not thermogenesis.* eLife, 2018. **7**: e32656.
77. Steneberg, P., Lindahl, E., Dahl, U., Lidh, E., Straseviciene, J., Backlund, F., Kjellkvist, E., Berggren, E., Lundberg, I., Bergqvist, I., Ericsson, M., Eriksson, B., Linde, K., Westman, J., Edlund, T., Edlund, H., *PAN-AMPK activator O304 improves glucose homeostasis and microvascular perfusion in mice and type 2 diabetes patients.* JCI Insight, 2018. **3**(12).
78. Inoki, K., Zhu, T., Guan, K.L., *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): 577-90.
79. Hardie, D.G., *AMPK and autophagy get connected.* EMBO J, 2011. **30**(4): 634-635.
80. Muslin, A.J., *MAPK Signaling in Cardiovascular Health and Disease: Molecular Mechanisms and Therapeutic Targets.* Clin Sci (Lond), 2008. **115**(7): 203-218.
81. Huang, A., Yang, Y.-M., Yan, C., Kaley, G., Hintze, T.H., Sun, D., *Altered MAPK Signaling in Progressive Deterioration of Endothelial Function in Diabetic Mice.* Diabetes, 2012. **61**(12): 3181-3188.
82. Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., *Polyphenols: food sources and bioavailability.* Am J Clin Nutr, 2004. **79**(5): 727-747.
83. Cory, H., Passarelli, S., Szeto, J., Tamez, M., Mattei, J., *The Role of Polyphenols in Human Health and Food Systems: A Mini-Review.* Front Nutr, 2018. **5**(87).
84. Williamson, G., Kay, C.D., Crozier, A., *The Bioavailability, Transport, and Bioactivity of Dietary Flavonoids: A Review from a Historical Perspective.* Compr Rev Food Sci Food Saf, 2018. **17**(5): 1054-1112.
85. Xiao, J.B., Hogger, P., *Dietary polyphenols and type 2 diabetes: current insights and future perspectives.* Curr Med Chem, 2015. **22**(1): 23-38.
86. Sharma, A., Kaur, M., Katnoria, J.K., Nagpal, A.K., *Polyphenols in Food: Cancer Prevention and Apoptosis Induction.* Curr Med Chem, 2017.

87. McCullough, M.L., Peterson, J.J., Patel, R., Jacques, P.F., Shah, R., Dwyer, J.T., *Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults.* Am J Clin Nutr, 2012. **95**(2): 454-64.
88. Elmann, A., Wang, C.-K., Vauzour, D., *Polyphenols Targeting Brain Cells Longevity, Brain's Redox Status, and Neurodegenerative Diseases.* Oxid Med Cell Longev, 2018. **2018**: 7402795-7402795.
89. Zhang, H., Tsao, R., *Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects.* Curr Opin Food Sci, 2016. **8**: 33-42.
90. Pandey, K.B., Rizvi, S.I., *Plant polyphenols as dietary antioxidants in human health and disease.* Oxid Med Cell Longev, 2009. **2**(5): 270-8.
91. Goszcz, K., Duthie, G.G., Stewart, D., Leslie, S.J., Megson, I.L., *Bioactive polyphenols and cardiovascular disease: chemical antagonists, pharmacological agents or xenobiotics that drive an adaptive response?* Br J Pharmacol, 2017. **174**(11): 1209-1225.
92. Tsao, R., *Chemistry and Biochemistry of Dietary Polyphenols.* Nutrients, 2010. **2**(12): 1231-1246.
93. Shen, T., Xie, C.-F., Wang, X.-N., Lou, H.-X., *Stilbenoids*, in *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*, K.G. Ramawat and J.-M. Mérillon, Editors. 2013, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 1901-1949.
94. Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., McCullough, M.L., *Dietary lignans: physiology and potential for cardiovascular disease risk reduction.* Nutr Rev, 2010. **68**(10): 571-603.
95. Shoji, T., Yamada, M., Miura, T., Nagashima, K., Ogura, K., Inagaki, N., Maeda-Yamamoto, M., *Chronic administration of apple polyphenols ameliorates hyperglycaemia in high-normal and borderline subjects: A randomised, placebo-controlled trial.* Diabetes Res Clin Pract, 2017. **129**: 43-51.
96. Rienks, J., Barbaresko, J., Oluwagbemigun, K., Schmid, M., Nothlings, U., *Polyphenol exposure and risk of type 2 diabetes: dose-response meta-analyses and systematic review of prospective cohort studies.* Am J Clin Nutr, 2018. **108**(1): 49-61.
97. Cao, H., Ou, J., Chen, L., Zhang, Y., Szkudelski, T., Delmas, D., Daglia, M., Xiao, J., *Dietary polyphenols and type 2 diabetes: Human study and clinical trials.* Crit Rev Food Sci Nutr, 2018: 1-19.
98. Song, Y., Manson, J.E., Buring, J.E., Sesso, H.D., Liu, S., *Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: a prospective study and cross-sectional analysis.* J Am Coll Nutr, 2005. **24**(5): 376-84.
99. Castro-Barquero, S., Lamuela-Raventós, R., Doménech, M., Estruch, R., *Relationship between Mediterranean Dietary Polyphenol Intake and Obesity.* Nutrients, 2018. **10**(10): 1523.
100. Carlstrom, M., Larsson, S.C., *Coffee consumption and reduced risk of developing type 2 diabetes: a systematic review with meta-analysis.* Nutr Rev, 2018. **76**(6): 395-417.
101. Alkhatib, A., Tsang, C., Tiss, A., Bahorun, T., Arefanian, H., Barake, R., Khadir, A., Tuomilehto, J., *Functional Foods and Lifestyle Approaches for Diabetes Prevention and Management.* Nutrients, 2017. **9**(12): 1310.
102. Ahrens, M.J., Thompson, D.L., *Effect of emulin on blood glucose in type 2 diabetics.* J Med Food, 2013. **16**(3): 211-5.
103. Rasouli, H., Hosseini-Ghazvini, S.M., Adibi, H., Khodarahmi, R., *Differential alpha-amylase/alpha-glucosidase inhibitory activities of plant-derived phenolic compounds: a virtual screening perspective for the treatment of obesity and diabetes.* Food Funct, 2017. **8**(5): 1942-1954.
104. Tsang, C., Smail, N.F., Almoosawi, S., Davidson, I., Al-Dujaili, E.A., *Intake of polyphenol-rich pomegranate pure juice influences urinary glucocorticoids, blood pressure and homeostasis model assessment of insulin resistance in human volunteers.* J Nutr Sci, 2012. **1**: e9.
105. Grassi, D., Desideri, G., Necozione, S., Lippi, C., Casale, R., Properzi, G., Blumberg, J.B., Ferri, C., *Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate.* J Nutr, 2008. **138**(9): 1671-6.
106. Brown, A.L., Lane, J., Coverly, J., Stocks, J., Jackson, S., Stephen, A., Bluck, L., Coward, A., Hendrickx, H., *Effects of dietary supplementation with the green tea polyphenol epigallocatechin-3-gallate on insulin resistance and associated metabolic risk factors: randomized controlled trial.* Br J Nutr, 2009. **101**(6): 886-94.
107. Chuang, C.C., Martinez, K., Xie, G., Kennedy, A., Bumrungpert, A., Overman, A., Jia, W., McIntosh, M.K., *Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-{alpha}-mediated inflammation and insulin resistance in primary human adipocytes.* Am J Clin Nutr, 2010. **92**(6): 1511-21.
108. Rhee, S.Y., Kim, Y.S., *The Role of Advanced Glycation End Products in Diabetic Vascular Complications.* Diabetes Metab J, 2018. **42**(3): 188-195.

109. Xu, B., Ji, Y., Yao, K., Cao, Y.X., Ferro, A., *Inhibition of human endothelial cell nitric oxide synthesis by advanced glycation end-products but not glucose: relevance to diabetes.* Clin Sci (Lond), 2005. **109**(5): 439-46.
110. Yeh, W.J., Hsia, S.M., Lee, W.H., Wu, C.H., *Polyphenols with antiglycation activity and mechanisms of action: A review of recent findings.* J Food Drug Anal, 2017. **25**(1): 84-92.
111. Fontana, L., *Interventions to promote cardiometabolic health and slow cardiovascular ageing.* Nat Rev Cardiol, 2018. **15**(9): 566-577.
112. Wang, X., Ouyang, Y.Y., Liu, J., Zhao, G., *Flavonoid intake and risk of CVD: a systematic review and meta-analysis of prospective cohort studies.* Br J Nutr, 2014. **111**(1): 1-11.
113. Tresserra-Rimbau, A., Rimm, E.B., Medina-Remon, A., Martinez-Gonzalez, M.A., de la Torre, R., Corella, D., Salas-Salvado, J., Gomez-Gracia, E., Lapetra, J., Aros, F., Fiol, M., Ros, E., Serra-Majem, L., Pinto, X., Saez, G.T., Basora, J., Sorli, J.V., Martinez, J.A., Vinyoles, E., Ruiz-Gutierrez, V., Estruch, R., Lamuela-Raventos, R.M., *Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study.* Nutr Metab Cardiovasc Dis, 2014. **24**(6): 639-47.
114. Pedret, A., Fernandez-Castillejo, S., Valls, R.M., Catalan, U., Rubio, L., Romeu, M., Macia, A., Lopez de Las Hazas, M.C., Farras, M., Giralt, M., Mosele, J.I., Martin-Pelaez, S., Remaley, A.T., Covas, M.I., Fito, M., Motilva, M.J., Sola, R., *Cardiovascular Benefits of Phenol-Enriched Virgin Olive Oils: New Insights from the Virgin Olive Oil and HDL Functionality (VOHF) Study.* Mol Nutr Food Res, 2018. **62**(16): e1800456.
115. Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., Kromhout, D., *Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study.* Lancet, 1993. **342**(8878): 1007-11.
116. Cassidy, A., Mukamal, K.J., Liu, L., Franz, M., Eliassen, A.H., Rimm, E.B., *High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women.* Circulation, 2013. **127**(2): 188-96.
117. Cassidy, A., Rimm, E.B., O'Reilly, E.J., Logroscino, G., Kay, C., Chiuve, S.E., Rexrode, K.M., *Dietary flavonoids and risk of stroke in women.* Stroke, 2012. **43**(4): 946-51.
118. Davies, J.M.S., Cillard, J., Friguet, B., Cadenas, E., Cadet, J., Cayce, R., Fishmann, A., Liao, D., Bulteau, A.-L., Derbré, F., Rébillard, A., Burstein, S., Hirsch, E., Kloner, R.A., Jakowec, M., Petzinger, G., Sauce, D., Sennlaub, F., Limon, I., Ursini, F., Maiorino, M., Economides, C., Pike, C.J., Cohen, P., Salvayre, A.N., Halliday, M.R., Lundquist, A.J., Jakowec, N.A., Mechta-Grigoriou, F., Mericskay, M., Mariani, J., Li, Z., Huang, D., Grant, E., Forman, H.J., Finch, C.E., Sun, P.Y., Pomatto, L.C.D., Agbulut, O., Warburton, D., Neri, C., Rouis, M., Cillard, P., Capeau, J., Rosenbaum, J., Davies, K.J.A., *The Oxygen Paradox, the French Paradox, and age-related diseases.* GeroScience, 2017. **39**(5-6): 499-550.
119. Stoclet, J.C., Chataigneau, T., Ndiaye, M., Oak, M.H., El Bedoui, J., Chataigneau, M., Schini-Kerth, V.B., *Vascular protection by dietary polyphenols.* Eur J Pharmacol, 2004. **500**(1-3): 299-313.
120. Auger, C., Teissedre, P.L., Gerain, P., Lequeux, N., Bornet, A., Serisier, S., Besancon, P., Caporiccio, B., Cristol, J.P., Rouanet, J.M., *Dietary wine phenolics catechin, quercetin, and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation.* J Agric Food Chem, 2005. **53**(6): 2015-21.
121. Yoshichika, K., *Understanding metabolic conversions and molecular actions of flavonoids in vivo:toward new strategies for effective utilization of natural polyphenols in human health.* J Med Invest, 2018. **65**(3.4): 162-165.
122. Itsiopoulos, C., Kucianski, T., Mayr, H.L., van Gaal, W.J., Martinez-Gonzalez, M.A., Vally, H., Kingsley, M., Kouris-Blazos, A., Radcliffe, J., Segal, L., Brazionis, L., Salim, A., Tierney, A.C., O'Dea, K., Wilson, A., Thomas, C.J., *The AUStralian MEDiterranean Diet Heart Trial (AUSMED Heart Trial): A randomized clinical trial in secondary prevention of coronary heart disease in a multiethnic Australian population: Study protocol.* Am Heart J, 2018. **203**: 4-11.
123. Noad, R.L., Rooney, C., McCall, D., Young, I.S., McCance, D., McKinley, M.C., Woodside, J.V., McKeown, P.P., *Beneficial effect of a polyphenol-rich diet on cardiovascular risk: a randomised control trial.* Heart, 2016. **102**(17): 1371-9.
124. Malik, A., Netticadan, T., Cordova, F., *Resveratrol: a Potential Anti-remodeling Agent in Heart Failure, From Bench to Bedside*. 2016, St. Boniface General Hospital Research Centre: https://clinicaltrials.gov/ct2/show/NCT01914081 (ClinicalTrials.gov).
125. Vetrani, C., Vitale, M., Bozzetto, L., Della Pepa, G., Cocozza, S., Costabile, G., Mangione, A., Cipriano, P., Annuzzi, G., Rivellese, A.A., *Association between different dietary polyphenol subclasses and the improvement in cardiometabolic risk factors: evidence from a randomized controlled clinical trial.* Acta Diabetol, 2018. **55**(2): 149-153.

126. Desch, S., Schmidt, J., Kobler, D., Sonnabend, M., Eitel, I., Sareban, M., Rahimi, K., Schuler, G., Thiele, H., *Effect of Cocoa Products on Blood Pressure: Systematic Review and Meta-Analysis.* Am J Hypertens, 2010. **23**(1): 97-103.
127. Egert, S., Bosy-Westphal, A., Seiberl, J., Kurbitz, C., Settler, U., Plachta-Danielzik, S., Wagner, A.E., Frank, J., Schrezenmeir, J., Rimbach, G., Wolffram, S., Muller, M.J., *Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study.* Br J Nutr, 2009. **102**(7): 1065-74.
128. Biesinger, S., Michaels, H.A., Quadros, A.S., Qian, Y., Rabovsky, A.B., Badger, R.S., Jalili, T., *A combination of isolated phytochemicals and botanical extracts lowers diastolic blood pressure in a randomized controlled trial of hypertensive subjects.* Eur J Clin Nutr, 2016. **70**(1): 10-6.
129. Barona, J., Aristizabal, J.C., Blesso, C.N., Volek, J.S., Fernandez, M.L., *Grape polyphenols reduce blood pressure and increase flow-mediated vasodilation in men with metabolic syndrome.* J Nutr, 2012. **142**(9): 1626-32.
130. Fogacci, F., Tocci, G., Presta, V., Fratter, A., Borghi, C., Cicero, A.F.G., *Effect of resveratrol on blood pressure: A systematic review and meta-analysis of randomized, controlled, clinical trials.* Crit Rev Food Sci Nutr, 2018: 1-14.
131. Hugel, H.M., Jackson, N., May, B., Zhang, A.L., Xue, C.C., *Polyphenol protection and treatment of hypertension.* Phytomedicine, 2016. **23**(2): 220-31.
132. Perez-Vizcaino, F., Duarte, J., Jimenez, R., Santos-Buelga, C., Osuna, A., *Antihypertensive effects of the flavonoid quercetin.* Pharmacol Rep, 2009. **61**(1): 67-75.
133. Suganya, N., Dornadula, S., Chatterjee, S., Mohanram, R.K., *Quercetin improves endothelial function in diabetic rats through inhibition of endoplasmic reticulum stress-mediated oxidative stress.* Eur J Pharmacol, 2018. **819**: 80-88.
134. Zhang, X.-H., Yokoo, H., Nishioka, H., Fujii, H., Matsuda, N., Hayashi, T., Hattori, Y., *Beneficial effect of the oligomerized polyphenol oligonol on high glucose-induced changes in eNOS phosphorylation and dephosphorylation in endothelial cells.* Br J Pharmacol, 2010. **159**(4): 928-938.
135. Ramirez-Sanchez, I., Aguilar, H., Ceballos, G., Villarreal, F., *(-)-Epicatechin-induced calcium independent eNOS activation: roles of HSP90 and AKT.* Mol Cell Biochem, 2012. **370**(1-2): 141-50.
136. Engler, M.B., Engler, M.M., *The emerging role of flavonoid-rich cocoa and chocolate in cardiovascular health and disease.* Nutr Rev, 2006. **64**(3): 109-18.
137. Nishida, S., Satoh, H., *Possible Involvement of Ca Activated K Channels, SK Channel, in the Quercetin-Induced Vasodilatation.* Korean J Physiol Pharmacol, 2009. **13**(5): 361-5.
138. Storniolo, C.E., Rosello-Catafau, J., Pinto, X., Mitjavila, M.T., Moreno, J.J., *Polyphenol fraction of extra virgin olive oil protects against endothelial dysfunction induced by high glucose and free fatty acids through modulation of nitric oxide and endothelin-1.* Redox Biol, 2014. **2**: 971-7.
139. Hettihewa, S., Hemar, Y., Rupasinghe, H., *Flavonoid-Rich Extract of Actinidia macrosperma (A Wild Kiwifruit) Inhibits Angiotensin-Converting Enzyme In Vitro.* Foods, 2018. **7**(9): 146.
140. Uhlenhut, K., Hogger, P., *Facilitated cellular uptake and suppression of inducible nitric oxide synthase by a metabolite of maritime pine bark extract (Pycnogenol).* Free Radic Biol Med, 2012. **53**(2): 305-13.
141. Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M.C.B., Rahu, N., *Oxidative Stress and Inflammation: What Polyphenols Can Do for Us?* Oxid Med Cell Longev, 2016. **2016**: 7432797-7432797.
142. Tangney, C.C., Rasmussen, H.E., *Polyphenols, inflammation, and cardiovascular disease.* Curr Atheroscler Rep, 2013. **15**(5): 324-324.
143. Hubbard, G.P., Wolffram, S., Lovegrove, J.A., Gibbins, J.M., *Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans.* J Thromb Haemost, 2004. **2**(12): 2138-45.
144. Wright, B., Spencer, J.P., Lovegrove, J.A., Gibbins, J.M., *Insights into dietary flavonoids as molecular templates for the design of anti-platelet drugs.* Cardiovasc Res, 2013. **97**(1): 13-22.
145. Sokoloff, B., Eddy, W.H., Redd, J.B., *The Biological Activity of a Flavonoid (Vitamin "P") Compound.* J Clin Invest, 1951. **30**(4): 395-400.
146. Belcaro, G., Dugall, M., Luzzi, R., Corsi, M., Ledda, A., Ricci, A., Pellegrini, L., Cesarone, M.R., Hosoi, M., Errichi, B.M., Cornelli, U., Cotellese, R., Agus, G., Feragalli, B., *Management of Varicose Veins and Chronic Venous Insufficiency in a Comparative Registry with Nine Venoactive Products in Comparison with Stockings.* Int J Angiol, 2017. **26**(3): 170-178.
147. Tahergorabi, Z., Khazaei, M., *Imbalance of angiogenesis in diabetic complications: the mechanisms.* Int J Prev Med, 2012. **3**(12): 827-838.
148. Soares, R., Costa, C., *Oxidative Stress, Inflammation and Angiogenesis in the Metabolic Syndrome*. 2009: Springer Netherlands. 85-99.

149. Williamson, G., Holst, B., *Dietary reference intake (DRI) value for dietary polyphenols: are we heading in the right direction?* Br J Nutr, 2008. **99 Suppl 3**: S55-8.
150. Navarro, V.J., Khan, I., Björnsson, E., Seeff, L.B., Serrano, J., Hoofnagle, J.H., *Liver injury from herbal and dietary supplements.* Hepatology, 2017. **65**(1): 363-373.
151. Martin, K., L Appel, C., *Polyphenols as dietary supplements: A double-edged sword.* Nutr Diet Suppl, 2009. **2**: 1-12.
152. Salehi, B., Mishra, A.P., Nigam, M., Sener, B., Kilic, M., Sharifi-Rad, M., Fokou, P.V.T., Martins, N., Sharifi-Rad, J., *Resveratrol: A Double-Edged Sword in Health Benefits.* Biomedicines, 2018. **6**(3): 91.
153. Boots, A.W., Li, H., Schins, R.P., Duffin, R., Heemskerk, J.W., Bast, A., Haenen, G.R., *The quercetin paradox.* Toxicol Appl Pharmacol, 2007. **222**(1): 89-96.
154. Harwood, M., Danielewska-Nikiel, B., Borzelleca, J.F., Flamm, G.W., Williams, G.M., Lines, T.C., *A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties.* Food Chem Toxicol, 2007. **45**(11): 2179-205.
155. Bacciottini, L., Falchetti, A., Pampaloni, B., Bartolini, E., Carossino, A.M., Brandi, M.L., *Phytoestrogens: food or drug?* Clin Cases Miner Bone Metab, 2007. **4**(2): 123-130.
156. *Risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones.* EFSA J, 2015. **13**(10): 4246.
157. Mazzanti, G., Di Sotto, A., Vitalone, A., *Hepatotoxicity of green tea: an update.* Arch Toxicol, 2015. **89**(8): 1175-91.
158. Younes, M., Aggett, P., Aguilar, F., Crebelli, R., Dusemund, B., Filipič, M., Frutos, M.J., Galtier, P., Gott, D., Gundert-Remy, U., Lambré, C., Leblanc, J.-C., Lillegaard, I.T., Moldeus, P., Mortensen, A., Oskarsson, A., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R.A., Andrade, R.J., Fortes, C., Mosesso, P., Restani, P., Arcella, D., Pizzo, F., Smeraldi, C., Wright, M., *Scientific opinion on the safety of green tea catechins.* EFSA J, 2018. **16**(4): e05239.
159. Ferry, D.R., Smith, A., Malkhandi, J., Fyfe, D.W., deTakats, P.G., Anderson, D., Baker, J., Kerr, D.J., *Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition.* Clin Cancer Res, 1996. **2**(4): 659-68.
160. Turner, R.S., Thomas, R.G., Craft, S., van Dyck, C.H., Mintzer, J., Reynolds, B.A., Brewer, J.B., Rissman, R.A., Raman, R., Aisen, P.S., *A randomized, double-blind, placebo-controlled trial of resveratrol for Alzheimer disease.* Neurology, 2015. **85**(16): 1383-91.
161. Liu, Y., Luo, X., Yang, C., Yang, T., Zhou, J., Shi, S., *Impact of quercetin‐induced changes in drug‐metabolizing enzyme and transporter expression on the pharmacokinetics of cyclosporine in rats.* Mol Med Report, 2016. **14**(4): 3073-3085.
162. Vrolijk, M.F., Haenen, G.R., Opperhuizen, A., Jansen, E.H., Schiffers, P.M., Bast, A., *The supplement-drug interaction of quercetin with tamsulosin on vasorelaxation.* Eur J Pharmacol, 2015. **746**: 132-7.
163. Ingolfsson, H.I., Thakur, P., Herold, K.F., Hobart, E.A., Ramsey, N.B., Periole, X., de Jong, D.H., Zwama, M., Yilmaz, D., Hall, K., Maretzky, T., Hemmings, H.C., Jr., Blobel, C., Marrink, S.J., Kocer, A., Sack, J.T., Andersen, O.S., *Phytochemicals perturb membranes and promiscuously alter protein function.* ACS Chem Biol, 2014. **9**(8): 1788-98.
164. Boly, R., Gras, T., Lamkami, T., Guissou, P., Serteyn, D., Kiss, R., Dubois, J., *Quercetin inhibits a large panel of kinases implicated in cancer cell biology.* Int J Oncol, 2011. **38**(3): 833-42.
165. Grimm, T., Skrabala, R., Chovanova, Z., Muchova, J., Sumegova, K., Liptakova, A., Durackova, Z., Hogger, P., *Single and multiple dose pharmacokinetics of maritime pine bark extract (pycnogenol) after oral administration to healthy volunteers.* BMC Clin Pharmacol, 2006. **6**: 4.
166. Packer, L., Rimbach, G., Virgili, F., *Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol.* Free Radic Biol Med, 1999. **27**(5-6): 704-24.
167. Liu, X., Zhou, H.J., Rohdewald, P., *French maritime pine bark extract Pycnogenol dose-dependently lowers glucose in type 2 diabetic patients.* Diabetes Care, 2004. **27**(3): 839.
168. Liu, X., Wei, J., Tan, F., Zhou, S., Wurthwein, G., Rohdewald, P., *Antidiabetic effect of Pycnogenol French maritime pine bark extract in patients with diabetes type II.* Life Sci, 2004. **75**(21): 2505-13.
169. Schafer, A., Hogger, P., *Oligomeric procyanidins of French maritime pine bark extract (Pycnogenol) effectively inhibit alpha-glucosidase.* Diabetes Res Clin Pract, 2007. **77**(1): 41-6.
170. Cesarone, M.R., Belcaro, G., Rohdewald, P., Pellegrini, L., Ledda, A., Vinciguerra, G., Ricci, A., Gizzi, G., Ippolito, E., Fano, F., Dugall, M., Cipollone, G., Acerbi, G., Cacchio, M., Del Boccio, G., Di Renzo, A., Stuard, S., Corsi, M., *Improvement of diabetic microangiopathy with pycnogenol: A prospective, controlled study.* Angiology, 2006. **57**(4): 431-6.

171. Steigerwalt, R., Belcaro, G., Cesarone, M.R., Di Renzo, A., Grossi, M.G., Ricci, A., Dugall, M., Cacchio, M., Schonlau, F., *Pycnogenol improves microcirculation, retinal edema, and visual acuity in early diabetic retinopathy.* J Ocul Pharmacol Ther, 2009. **25**(6): 537-40.
172. Spadea, L., Balestrazzi, E., *Treatment of vascular retinopathies with Pycnogenol.* Phytother Res, 2001. **15**(3): 219-23.
173. Cesarone, M.R., Belcaro, G., Stuard, S., Schonlau, F., Di Renzo, A., Grossi, M.G., Dugall, M., Cornelli, U., Cacchio, M., Gizzi, G., Pellegrini, L., *Kidney flow and function in hypertension: protective effects of pycnogenol in hypertensive participants--a controlled study.* J Cardiovasc Pharmacol Ther, 2010. **15**(1): 41-6.
174. Belcaro, G., Cornelli, U., Luzzi, R., Cesarone, M.R., Dugall, M., Feragalli, B., Errichi, S., Ippolito, E., Grossi, M.G., Hosoi, M., Cornelli, M., Gizzi, G., *Pycnogenol(R) supplementation improves health risk factors in subjects with metabolic syndrome.* Phytother Res, 2013. **27**(10): 1572-8.
175. Belcaro, G., Dugall, M., Hu, S., Feragalli, B., Cotellese, R., Ledda, A., Corsi, M., Ricci, A., Ippolito, E., Errichi, B.M., Cornelli, U., Cesarone, M.R., Hosoi, M., *Prevention of recurrent venous thrombosis and post-thrombotic syndrome.* Minerva Cardioangiol, 2018. **66**(3): 238-245.
176. Rohdewald, P., *Pleiotropic Effects of French Maritime Pine Bark Extract to Promote Healthy Aging.* Rejuvenation Res, 2018.
177. Mülek, M., Seefried, L., Genest, F., Högger, P., *Distribution of Constituents and Metabolites of Maritime Pine Bark Extract (Pycnogenol®) into Serum, Blood Cells, and Synovial Fluid of Patients with Severe Osteoarthritis: A Randomized Controlled Trial.* Nutrients, 2017. **9**(5): 443.
178. Duweler, K.G., Rohdewald, P., *Urinary metabolites of French maritime pine bark extract in humans.* Pharmazie, 2000. **55**(5): 364-8.
179. Belcaro, G., Hu, S., Cesarone, M.R., Dugall, M., *A controlled study shows daily intake of 50 mg of French Pine Bark Extract (Pycnogenol(R)) lowers plasma reactive oxygen metabolites in healthy smokers.* Minerva Med, 2013. **104**(4): 439-46.
180. Grimm, T., Schafer, A., Hogger, P., *Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol).* Free Radic Biol Med, 2004. **36**(6): 811-22.
181. Schafer, A., Chovanova, Z., Muchova, J., Sumegova, K., Liptakova, A., Durackova, Z., Hogger, P., *Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol).* Biomed Pharmacother, 2006. **60**(1): 5-9.
182. Gulati, O.P., *Pycnogenol® in Metabolic Syndrome and Related Disorders.* Phytother Res, 2015. **29**(7): 949-968.
183. Providencia, R., *Cardiovascular protection from alcoholic drinks: scientific basis of the French Paradox.* Rev Port Cardiol, 2006. **25**(11): 1043-58.
184. Lekakis, J., Rallidis, L.S., Andreadou, I., Vamvakou, G., Kazantzoglou, G., Magiatis, P., Skaltsounis, A.L., Kremastinos, D.T., *Polyphenolic compounds from red grapes acutely improve endothelial function in patients with coronary heart disease.* Eur J Cardiovasc Prev Rehabil, 2005. **12**(6): 596-600.
185. Rodrigo, R., Gil, D., Miranda-Merchak, A., Kalantzidis, G., *Antihypertensive role of polyphenols.* Adv Clin Chem, 2012. **58**: 225-54.
186. de Brito Alves, J.L., de Sousa, V.P., Cavalcanti Neto, M.P., Magnani, M., Braga, V.A., da Costa-Silva, J.H., Leandro, C.G., Vidal, H., Pirola, L., *New Insights on the Use of Dietary Polyphenols or Probiotics for the Management of Arterial Hypertension.* Front Physiol, 2016. **7**: 448.
187. Lim, G.E., Brubaker, P.L., *Glucagon-Like Peptide 1 Secretion by the L-Cell: The View From Within.* Diabetes, 2006. **55**(Supplement 2): S70-S77.
188. Campbell, J.E., Drucker, D.J., *Pharmacology, physiology, and mechanisms of incretin hormone action.* Cell Metab, 2013. **17**(6): 819-37.
189. Oyama, J., Higashi, Y., Node, K., *Do incretins improve endothelial function?* Cardiovasc Diabetol, 2014. **13**(1): 1-8.
190. Ban, K., Noyan-Ashraf, M.H., Hoefer, J., Bolz, S.S., Drucker, D.J., Husain, M., *Cardioprotective and Vasodilatory Actions of Glucagon-Like Peptide 1 Receptor Are Mediated Through Both Glucagon-Like Peptide 1 Receptor–Dependent and –Independent Pathways.* Circulation, 2008. **117**(18): 2340-2350.
191. Frigge, M., Hoaglin, D.C., Iglewicz, B., *Some Implementations of the Boxplot.* Am Stat, 1989. **43**(1): 50-54.
192. Grubbs, F.E., *Procedures for Detecting Outlying Observations in Samples.* Technometrics, 1969. **11**(1): 1-21.
193. Torronen, R., Sarkkinen, E., Niskanen, T., Tapola, N., Kilpi, K., Niskanen, L., *Postprandial glucose, insulin and glucagon-like peptide 1 responses to sucrose ingested with berries in healthy subjects.* Br J Nutr, 2012. **107**(10): 1445-51.

194. Elliott, R.M., Morgan, L.M., Tredger, J.A., Deacon, S., Wright, J., Marks, V., *Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns.* J Endocrinol, 1993. **138**(1): 159-66.
195. Ryskjær, J., Deacon, C.F., Carr, R.D., Krarup, T., Madsbad, S., Holst, J., Vilsbøll, T., *Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbAlc levels, but is not acutely affected by food intake.* Eur J Endocrinol, 2006. **155**(3): 485-493.
196. Vilsboll, T., Krarup, T., Deacon, C.F., Madsbad, S., Holst, J.J., *Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients.* Diabetes, 2001. **50**(3): 609-13.
197. Hillman, M., Eriksson, L., Mared, L., Helgesson, K., Landin-Olsson, M., *Reduced levels of active GLP-1 in patients with cystic fibrosis with and without diabetes mellitus.* J Cyst Fibros, 2012. **11**(2): 144-9.
198. Holst, J.J., *The physiology of glucagon-like peptide 1*. Physiol Rev, 2007. **87**(4): 1409-39.
199. Faerch, K., Torekov, S.S., Vistisen, D., Johansen, N.B., Witte, D.R., Jonsson, A., Pedersen, O., Hansen, T., Lauritzen, T., Sandbaek, A., Holst, J.J., Jorgensen, M.E., *GLP-1 Response to Oral Glucose Is Reduced in Prediabetes, Screen-Detected Type 2 Diabetes, and Obesity and Influenced by Sex: The ADDITION-PRO Study.* Diabetes, 2015. **64**(7): 2513-25.
200. Mannucci, E., Ognibene, A., Cremasco, F., Bardini, G., Mencucci, A., Pierazzuoli, E., Ciani, S., Fanelli, A., Messeri, G., Rotella, C.M., *Glucagon-like peptide (GLP)-1 and leptin concentrations in obese patients with Type 2 diabetes mellitus.* Diabet Med, 2000. **17**(10): 713-9.
201. Seifarth, C., Bergmann, J., Holst, J.J., Ritzel, R., Schmiegel, W., Nauck, M.A., *Prolonged and enhanced secretion of glucagon-like peptide 1 (7-36 amide) after oral sucrose due to alpha-glucosidase inhibition (acarbose) in Type 2 diabetic patients.* Diabet Med, 1998. **15**(6): 485-91.
202. Hoggard, N., Cruickshank, M., Moar, K.M., Bestwick, C., Holst, J.J., Russell, W., Horgan, G., *A single supplement of a standardised bilberry (Vaccinium myrtillus L.) extract (36 % wet weight anthocyanins) modifies glycaemic response in individuals with type 2 diabetes controlled by diet and lifestyle.* J Nutr Sci, 2013. **2**: e22.
203. Johnston, K.L., Clifford, M.N., Morgan, L.M., *Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine.* Am J Clin Nutr, 2003. **78**(4): 728-33.
204. Lambeir, A.M., Durinx, C., Scharpe, S., De Meester, I., *Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV.* Crit Rev Clin Lab Sci, 2003. **40**(3): 209-94.
205. Deacon, C.F., Lebovitz, H.E., *Comparative review of dipeptidyl peptidase-4 inhibitors and sulphonylureas.* Diabetes Obes Metab, 2016. **18**(4): 333-347.
206. Lamers, D., Famulla, S., Wronkowitz, N., Hartwig, S., Lehr, S., Ouwens, D.M., Eckardt, K., Kaufman, J.M., Ryden, M., Muller, S., Hanisch, F.G., Ruige, J., Arner, P., Sell, H., Eckel, J., *Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome.* Diabetes, 2011. **60**(7): 1917-25.
207. Cordero, O.J., Salgado, F.J., Mera-Varela, A., Nogueira, M., *Serum interleukin-12, interleukin-15, soluble CD26, and adenosine deaminase in patients with rheumatoid arthritis.* Rheumatol Int, 2001. **21**(2): 69-74.
208. Cordero, O.J., Salgado, F.J., Nogueira, M., *On the origin of serum CD26 and its altered concentration in cancer patients.* Cancer Immunol Immunother, 2009. **58**(11): 1723-47.
209. Chang, X.Y., Yang, Y., Jia, X.Q., Wang, Y., Peng, L.N., Ai, X.H., Jiang, C.Y., Guo, J.H., Wu, T.T., *Expression and Clinical Significance of Serum Dipeptidyl Peptidase IV Chronic Obstructive Pulmonary Disease.* Am J Med Sci, 2016. **351**(3): 244-52.
210. Buljevic, S., Detel, D., Pucar, L.B., Mihelic, R., Madarevic, T., Sestan, B., Varljen, J., *Levels of dipeptidyl peptidase IV/CD26 substrates neuropeptide Y and vasoactive intestinal peptide in rheumatoid arthritis patients.* Rheumatol Int, 2013. **33**(11): 2867-74.
211. Durinx, C., Lambeir, A.M., Bosmans, E., Falmagne, J.B., Berghmans, R., Haemers, A., Scharpe, S., De Meester, I., *Molecular characterization of dipeptidyl peptidase activity in serum: soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides.* Eur J Biochem, 2000. **267**(17): 5608-13.
212. Plamboeck, A., Holst, J.J., Carr, R.D., Deacon, C.F., *Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both mediators of the degradation of glucagon-like peptide 1 in the anaesthetised pig.* Diabetologia, 2005. **48**(9): 1882-90.
213. Rhee, N.A., Ostoft, S.H., Holst, J.J., Deacon, C.F., Vilsboll, T., Knop, F.K., *The impact of dipeptidyl peptidase 4 inhibition on incretin effect, glucose tolerance, and gastrointestinal-mediated glucose disposal in healthy subjects.* Eur J Endocrinol, 2014. **171**(3): 353-62.

214. Mannucci, E., Pala, L., Ciani, S., Bardini, G., Pezzatini, A., Sposato, I., Cremasco, F., Ognibene, A., Rotella, C.M., *Hyperglycaemia increases dipeptidyl peptidase IV activity in diabetes mellitus.* Diabetologia, 2005. **48**(6): 1168-72.
215. Fan, J., Johnson, M.H., Lila, M.A., Yousef, G., de Mejia, E.G., *Berry and Citrus Phenolic Compounds Inhibit Dipeptidyl Peptidase IV: Implications in Diabetes Management.* Evid Based Complement Alternat Med, 2013. **2013**: 479505.
216. Bansal, P., Paul, P., Mudgal, J., Nayak, P.G., Pannakal, S.T., Priyadarsini, K.I., Unnikrishnan, M.K., *Antidiabetic, antihyperlipidemic and antioxidant effects of the flavonoid rich fraction of Pilea microphylla (L.) in high fat diet/streptozotocin-induced diabetes in mice.* Exp Toxicol Pathol, 2012. **64**(6): 651-8.
217. Kosaraju, J., Dubala, A., Chinni, S., Khatwal, R.B., Satish Kumar, M.N., Basavan, D., *A molecular connection of Pterocarpus marsupium, Eugenia jambolana and Gymnema sylvestre with dipeptidyl peptidase-4 in the treatment of diabetes.* Pharm Biol, 2014. **52**(2): 268-71.
218. Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., *Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies.* Am J Clin Nutr, 2005. **81**(1 Suppl): 230s-242s.
219. Adolpho, L.O., Marin, D., Puigpinos, A., Mendieta, L., Tarrago, T., Morel, A.F., Giralt, E., Dalcol, II, *In vitro evaluation of caffeoyl and cinnamoyl derivatives as potential prolyl oligopeptidase inhibitors.* Planta Med, 2013. **79**(16): 1531-5.
220. Roškar, I., Štrukelj, B., Lunder, M., *Screening of Phenolic Compounds Reveals Inhibitory Activity of Nordihydroguaiaretic Acid Against Three Enzymes Involved in the Regulation of Blood Glucose Level.* Plant Foods Hum Nutr, 2016. **71**(1): 88-89.
221. Bower, A.M., Real Hernandez, L.M., Berhow, M.A., de Mejia, E.G., *Bioactive compounds from culinary herbs inhibit a molecular target for type 2 diabetes management, dipeptidyl peptidase IV.* J Agric Food Chem, 2014. **62**(26): 6147-58.
222. Kozuka, M., Yamane, T., Nakano, Y., Nakagaki, T., Ohkubo, I., Ariga, H., *Identification and characterization of a dipeptidyl peptidase IV inhibitor from aronia juice.* Biochem Biophys Res Commun, 2015. **465**(3): 433-6.
223. Szepietowska, B., Gorska, M., Szelachowska, M., *Plasma relaxin concentration is related to beta-cell function and insulin sensitivity in women with type 2 diabetes mellitus.* Diabetes Res Clin Pract, 2008. **79**(3): e1-3.
224. Zhang, X., Zhu, M., Zhao, M., Chen, W., Fu, Y., Liu, Y., Liu, W., Zhang, B., Yin, X., Bai, B., *The plasma levels of relaxin-2 and relaxin-3 in patients with diabetes.* Clin Biochem, 2013. **46**(16-17): 1713-6.
225. Whittaker, P.G., Edwards, J.R., Randolph, C., Bullesbach, E.E., Schwabe, C., Steinetz, B.G., *Abnormal relaxin secretion during pregnancy in women with type 1 diabetes.* Exp Biol Med (Maywood), 2003. **228**(1): 33-40.
226. Wiecek, A., Kokot, F., Chudek, J., Adamczak, M., *The adipose tissue--a novel endocrine organ of interest to the nephrologist.* Nephrol Dial Transplant, 2002. **17**(2): 191-5.
227. Ukkola, O., Santaniemi, M., *Adiponectin: a link between excess adiposity and associated comorbidities?* J Mol Med (Berl), 2002. **80**(11): 696-702.
228. Yamauchi, T., Kadowaki, T., *Physiological and pathophysiological roles of adiponectin and adiponectin receptors in the integrated regulation of metabolic and cardiovascular diseases.* Int J Obes (Lond), 2008. **32 Suppl 7**: S13-8.
229. Ouchi, N., Kihara, S., Arita, Y., Okamoto, Y., Maeda, K., Kuriyama, H., Hotta, K., Nishida, M., Takahashi, M., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Funahashi, T., Matsuzawa, Y., *Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway.* Circulation, 2000. **102**(11): 1296-301.
230. Zoccali, C., Mallamaci, F., Tripepi, G., Benedetto, F.A., Cutrupi, S., Parlongo, S., Malatino, L.S., Bonanno, G., Seminara, G., Rapisarda, F., Fatuzzo, P., Buemi, M., Nicocia, G., Tanaka, S., Ouchi, N., Kihara, S., Funahashi, T., Matsuzawa, Y., *Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease.* J Am Soc Nephrol, 2002. **13**(1): 134-41.
231. Swarbrick, M.M., Havel, P.J., *Physiological, pharmacological, and nutritional regulation of circulating adiponectin concentrations in humans.* Metab Syndr Relat Disord, 2008. **6**(2): 87-102.
232. Okada-Iwabu, M., Iwabu, M., Ueki, K., Yamauchi, T., Kadowaki, T., *Perspective of Small-Molecule AdipoR Agonist for Type 2 Diabetes and Short Life in Obesity.* Diabetes Metab J, 2015. **39**(5): 363-72.
233. Lee, C.Y., Jan, M.S., Yu, M.C., Lin, C.C., Wei, J.C., Shih, H.C., *Relationship between adiponectin and leptin, and blood lipids in hyperlipidemia patients treated with red yeast rice.* Forsch Komplementmed, 2013. **20**(3): 197-203.

234. Lozano, A., Perez-Martinez, P., Marin, C., Tinahones, F.J., Delgado-Lista, J., Cruz-Teno, C., Gomez-Luna, P., Rodriguez-Cantalejo, F., Perez-Jimenez, F., Lopez-Miranda, J., *An acute intake of a walnut-enriched meal improves postprandial adiponectin response in healthy young adults.* Nutr Res, 2013. **33**(12): 1012-8.
235. Aronis, K.N., Vamvini, M.T., Chamberland, J.P., Sweeney, L.L., Brennan, A.M., Magkos, F., Mantzoros, C.S., *Short-term walnut consumption increases circulating total adiponectin and apolipoprotein A concentrations, but does not affect markers of inflammation or vascular injury in obese humans with the metabolic syndrome: data from a double-blinded, randomized, placebo-controlled study.* Metabolism, 2012. **61**(4): 577-82.
236. Sone, T., Kuriyama, S., Nakaya, N., Hozawa, A., Shimazu, T., Nomura, K., Rikimaru, S., Tsuji, I., *Randomized controlled trial for an effect of catechin-enriched green tea consumption on adiponectin and cardiovascular disease risk factors.* Food Nutr Res, 2011. **55**.
237. Tome-Carneiro, J., Gonzalvez, M., Larrosa, M., Yanez-Gascon, M.J., Garcia-Almagro, F.J., Ruiz-Ros, J.A., Tomas-Barberan, F.A., Garcia-Conesa, M.T., Espin, J.C., *Grape resveratrol increases serum adiponectin and downregulates inflammatory genes in peripheral blood mononuclear cells: a triple-blind, placebo-controlled, one-year clinical trial in patients with stable coronary artery disease.* Cardiovasc Drugs Ther, 2013. **27**(1): 37-48.
238. Wu, A.H., Spicer, D., Stanczyk, F.Z., Tseng, C.C., Yang, C.S., Pike, M.C., *Effect of 2-month controlled green tea intervention on lipoprotein cholesterol, glucose, and hormone levels in healthy postmenopausal women.* Cancer Prev Res (Phila), 2012. **5**(3): 393-402.
239. Liu, C.Y., Huang, C.J., Huang, L.H., Chen, I.J., Chiu, J.P., Hsu, C.H., *Effects of green tea extract on insulin resistance and glucagon-like peptide 1 in patients with type 2 diabetes and lipid abnormalities: a randomized, double-blinded, and placebo-controlled trial.* PLoS One, 2014. **9**(3): e91163.
240. Yamauchi, J., Osawa, H., Takasuka, T., Ochi, M., Murakami, A., Nishida, W., Onuma, H., Takata, Y., Tabara, Y., Miki, T., Ohashi, J., Makino, H., *Serum resistin is reduced by glucose and meal loading in healthy human subjects.* Metabolism, 2008. **57**(2): 149-56.
241. Karlsson, F.A., Engstrom, B.E., Lind, L., Ohrvall, M., *No postprandial increase of plasma adiponectin in obese subjects.* Obes Res, 2004. **12**(6): 1031-2; author reply 1032-4.
242. Ozeki, N., Hara, K., Yatsuka, C., Nakano, T., Matsumoto, S., Suetsugu, M., Nakamachi, T., Takebayashi, K., Inukai, T., Haruki, K., Aso, Y., *Serum high-molecular weight adiponectin decreases abruptly after an oral glucose load in subjects with normal glucose tolerance or impaired fasting glucose, but not those with impaired glucose tolerance or diabetes mellitus.* Metabolism, 2009. **58**(10): 1470-6.
243. Rubin, D., Helwig, U., Nothnagel, M., Lemke, N., Schreiber, S., Folsch, U.R., Doring, F., Schrezenmeir, J., *Postprandial plasma adiponectin decreases after glucose and high fat meal and is independently associated with postprandial triacylglycerols but not with -- 11388 promoter polymorphism.* Br J Nutr, 2008. **99**(1): 76-82.
244. Esposito, K., Nappo, F., Giugliano, F., Di Palo, C., Ciotola, M., Barbieri, M., Paolisso, G., Giugliano, D., *Meal modulation of circulating interleukin 18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus.* Am J Clin Nutr, 2003. **78**(6): 1135-40.
245. Horakova, D., Azeem, K., Benesova, R., Pastucha, D., Horak, V., Dumbrovska, L., Martinek, A., Novotny, D., Svagera, Z., *Total and High Molecular Weight Adiponectin Levels and Prediction of Cardiovascular Risk in Diabetic Patients.* J Endocrinol, 2015. **2015**: 545068.
246. Bluher, M., Brennan, A.M., Kelesidis, T., Kratzsch, J., Fasshauer, M., Kralisch, S., Williams, C.J., Mantzoros, C.S., *Total and high-molecular weight adiponectin in relation to metabolic variables at baseline and in response to an exercise treatment program: comparative evaluation of three assays.* Diabetes Care, 2007. **30**(2): 280-5.
247. Han, S.H., Sakuma, I., Shin, E.K., Koh, K.K., *Antiatherosclerotic and anti-insulin resistance effects of adiponectin: basic and clinical studies.* Prog Cardiovasc Dis, 2009. **52**(2): 126-40.
248. Zdychova, J., Komers, R., *Emerging role of Akt kinase/protein kinase B signaling in pathophysiology of diabetes and its complications.* Physiol Res, 2005. **54**(1): 1-16.
249. Kohn, A.D., Summers, S.A., Birnbaum, M.J., Roth, R.A., *Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation.* J Biol Chem, 1996. **271**(49): 31372-8.
250. Shiojima, I., Walsh, K., *Role of Akt signaling in vascular homeostasis and angiogenesis.* Circ Res, 2002. **90**(12): 1243-50.
251. Taguchi, K., Hida, M., Hasegawa, M., Matsumoto, T., Kobayashi, T., *Dietary polyphenol morin rescues endothelial dysfunction in a diabetic mouse model by activating the Akt/eNOS pathway.* Mol Nutr Food Res, 2016. **60**(3): 580-8.

252. Jang, H.J., Ridgeway, S.D., Kim, J.A., *Effects of the green tea polyphenol epigallocatechin-3-gallate on high-fat diet-induced insulin resistance and endothelial dysfunction.* Am J Physiol Endocrinol Metab, 2013. **305**(12): E1444-51.
253. Yang, D.K., Kang, H.S., *Anti-Diabetic Effect of Cotreatment with Quercetin and Resveratrol in Streptozotocin-Induced Diabetic Rats.* Biomol Ther (Seoul), 2018. **26**(2): 130-8.
254. Mellor, D.D., Madden, L.A., Smith, K.A., Kilpatrick, E.S., Atkin, S.L., *High-polyphenol chocolate reduces endothelial dysfunction and oxidative stress during acute transient hyperglycaemia in Type 2 diabetes: a pilot randomized controlled trial.* Diabet Med, 2013. **30**(4): 478-83.
255. Choi, E.Y., Lee, H., Woo, J.S., Jang, H.H., Hwang, S.J., Kim, H.S., Kim, W.S., Kim, Y.S., Choue, R., Cha, Y.J., Yim, J.E., Kim, W., *Effect of onion peel extract on endothelial function and endothelial progenitor cells in overweight and obese individuals.* Nutrition, 2015. **31**(9): 1131-5.
256. Li, D., Zhang, Y., Liu, Y., Sun, R., Xia, M., *Purified anthocyanin supplementation reduces dyslipidemia, enhances antioxidant capacity, and prevents insulin resistance in diabetic patients.* J Nutr, 2015. **145**(4): 742-8.
257. Li, J., Huang, W., Tu, R., Zhong, G., Luo, B., He, Y., *Resveratrol rescues hyperglycemia-induced endothelial dysfunction via activation of Akt.* Acta Pharmacol Sin, 2017. **38**(2): 182-91.
258. Zhang, H., Zhang, J., Ungvari, Z., Zhang, C., *Resveratrol Improves Endothelial Function: Role of TNFα and Vascular Oxidative Stress.* Arterioscler Thromb Vasc Biol, 2009. **29**(8): 1164-1171.
259. Xia, N., Forstermann, U., Li, H., *Resveratrol and endothelial nitric oxide.* Molecules, 2014. **19**(10): 16102-21.
260. Sanchez, M., Galisteo, M., Vera, R., Villar, I.C., Zarzuelo, A., Tamargo, J., Perez-Vizcaino, F., Duarte, J., *Quercetin downregulates NADPH oxidase, increases eNOS activity and prevents endothelial dysfunction in spontaneously hypertensive rats.* J Hypertens, 2006. **24**(1): 75-84.
261. Guo, X.D., Zhang, D.Y., Gao, X.J., Parry, J., Liu, K., Liu, B.L., Wang, M., *Quercetin and quercetin-3-O-glucuronide are equally effective in ameliorating endothelial insulin resistance through inhibition of reactive oxygen species-associated inflammation.* Mol Nutr Food Res, 2013. **57**(6): 1037-45.
262. Matter, W.F., Brown, R.F., Vlahos, C.J., *The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs.* Biochem Biophys Res Commun, 1992. **186**(2): 624-31.
263. Kampa-Schittenhelm, K.M., Heinrich, M.C., Akmut, F., Rasp, K.H., Illing, B., Döhner, H., Döhner, K., Schittenhelm, M.M., *Cell cycle-dependent activity of the novel dual PI3K-MTORC1/2 inhibitor NVP-BGT226 in acute leukemia.* Mol Cancer, 2013. **12**(1): 1-18.
264. Jackson, S.J., Venema, R.C., *Quercetin inhibits eNOS, microtubule polymerization, and mitotic progression in bovine aortic endothelial cells.* J Nutr, 2006. **136**(5): 1178-84.
265. Shakibaei, M., Harikumar, K.B., Aggarwal, B.B., *Resveratrol addiction: to die or not to die.* Mol Nutr Food Res, 2009. **53**(1): 115-28.
266. Kanavi, M.R., Darjatmoko, S., Wang, S., Azari, A.A., Farnoodian, M., Kenealey, J.D., van Ginkel, P.R., Albert, D.M., Sheibani, N., Polans, A.S., *The Sustained Delivery of Resveratrol or a Defined Grape Powder Inhibits New Blood Vessel Formation in a Mouse Model of Choroidal Neovascularization.* Molecules, 2014. **19**(11): 17578-603.
267. In, K., Park, J., Park, H., *Resveratrol at high doses acts as an apoptotic inducer in endothelial cells.* Cancer Res Treat, 2006. **38**(1): 48-53.
268. Takahashi, S., Uchiyama, T., Toda, K., *Differential effect of resveratrol on nitric oxide production in endothelial f-2 cells.* Biol Pharm Bull, 2009. **32**(11): 1840-3.
269. Dimmeler, S., Dernbach, E., Zeiher, A.M., *Phosphorylation of the endothelial nitric oxide synthase at Ser-1177 is required for VEGF-induced endothelial cell migration.* FEBS Lett, 2000. **477**(3): 258-262.
270. Pektas, M.B., Turan, O., Ozturk Bingol, G., Sumlu, E., Sadi, G., Akar, F., *High glucose causes vascular dysfunction through Akt/eNOS pathway: reciprocal modulation by juglone and resveratrol.* Can J Physiol Pharmacol, 2018: 1-8.
271. Simão, F., Pagnussat, A.S., Seo, J.H., Navaratna, D., Leung, W., Lok, J., Guo, S., Waeber, C., Salbego, C.G., Lo, E.H., *Pro-angiogenic effects of resveratrol in brain endothelial cells: nitric oxide-mediated regulation of vascular endothelial growth factor and metalloproteinases.* J Cereb Blood Flow Metab, 2012. **32**(5): 884-95.
272. Xiao, J., Hogger, P., *Stability of dietary polyphenols under the cell culture conditions: avoiding erroneous conclusions.* J Agric Food Chem, 2015. **63**(5): 1547-57.
273. Wilsher, N.E., Arroo, R.R., Matsoukas, M.T., Tsatsakis, A.M., Spandidos, D.A., Androutsopoulos, V.P., *Cytochrome P450 CYP1 metabolism of hydroxylated flavones and flavonols: Selective bioactivation of luteolin in breast cancer cells.* Food Chem Toxicol, 2017. **110**: 383-394.
274. Potter, G.A., Patterson, L.H., Wanogho, E., Perry, P.J., Butler, P.C., Ijaz, T., Ruparelia, K.C., Lamb, J.H., Farmer, P.B., Stanley, L.A., Burke, M.D., *The cancer preventative agent resveratrol is*

converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. Br J Cancer, 2002. **86**(5): 774-8.
275. Castillo-Pichardo, L., Martínez, E.J., *Inhibition of mammary tumor growth and metastases to bone and liver by dietary grape polyphenols.* Clin Exp Metastasis, 2009. **26**(6): 505-516.
276. Huang, S., Zhu, X., Huang, W., He, Y., Pang, L., Lan, X., Shui, X., Chen, Y., Chen, C., Lei, W., *Quercetin Inhibits Pulmonary Arterial Endothelial Cell Transdifferentiation Possibly by Akt and Erk1/2 Pathways.* Biomed Res Int, 2017. **2017**: 8.
277. Bagli, E., Stefaniotou, M., Morbidelli, L., Ziche, M., Psillas, K., Murphy, C., Fotsis, T., *Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity.* Cancer Res, 2004. **64**(21): 7936-46.
278. Erdogan, A., Most, A.K., Wienecke, B., Fehsecke, A., Leckband, C., Voss, R., Grebe, M.T., Tillmanns, H., Schaefer, C.A., Kuhlmann, C.R., *Apigenin-induced nitric oxide production involves calcium-activated potassium channels and is responsible for antiangiogenic effects.* J Thromb Haemost, 2007. **5**(8): 1774-81.
279. Gao, X., Liu, K., Huang, F., Zhang, D., Guo, X., Wang, M., Liu, B., *Positive and negative regulation of insulin action by genistein in the endothelium.* J Nutr Biochem, 2013. **24**(1): 222-30.
280. Vincent, E.E., Elder, D.J.E., Thomas, E.C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M.T., Hetzel, M.R., Tavaré, J.M., *Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer.* Br J Cancer, 2011. **104**(11): 1755-61.
281. Tremblay, F., Krebs, M., Dombrowski, L., Brehm, A., Bernroider, E., Roth, E., Nowotny, P., Waldhäusl, W., Marette, A., Roden, M., *Overactivation of S6 Kinase 1 as a Cause of Human Insulin Resistance During Increased Amino Acid Availability.* Diabetes, 2005. **54**(9): 2674-2684.
282. Rajapakse, A.G., Yepuri, G., Carvas, J.M., Stein, S., Matter, C.M., Scerri, I., Ruffieux, J., Montani, J.P., Ming, X.F., Yang, Z., *Hyperactive S6K1 mediates oxidative stress and endothelial dysfunction in aging: inhibition by resveratrol.* PLoS One, 2011. **6**(4): e19237.
283. Sheu, M.L., Ho, F.M., Yang, R.S., Chao, K.F., Lin, W.W., Lin-Shiau, S.Y., Liu, S.H., *High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway.* Arterioscler Thromb Vasc Biol, 2005. **25**(3): 539-45.
284. Heath, J.M., Sun, Y., Yuan, K., Bradley, W.E., Litovsky, S., Dell'Italia, L.J., Chatham, J.C., Wu, H., Chen, Y., *Activation of AKT by O-GlcNAcylation Induces Vascular Calcification in Diabetes.* Circ Res, 2014. **114**(7): 1094-1102.
285. Jiang, B.H., Liu, L.Z., *AKT signaling in regulating angiogenesis.* Curr Cancer Drug Targets, 2008. **8**(1): 19-26.
286. Costa, P.Z., Soares, R., *Neovascularization in diabetes and its complications. Unraveling the angiogenic paradox.* Life Sci, 2013. **92**(22): 1037-45.
287. Phung, T.L., Ziv, K., Dabydeen, D., Eyiah-Mensah, G., Riveros, M., Perruzzi, C., Sun, J., Monahan-Earley, R.A., Shiojima, I., Nagy, J.A., Lin, M.I., Walsh, K., Dvorak, A.M., Briscoe, D.M., Neeman, M., Sessa, W.C., Dvorak, H.F., Benjamin, L.E., *Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin.* Cancer Cell, 2006. **10**(2): 159-70.
288. Kim, Y., Lim, H., Lee, S., Shin, M., Kim, C., Yang, H.J., *Betaine inhibits vascularization via suppression of Akt in the retinas of streptozotocin-induced hyperglycemic rats.* Mol Med Rep, 2015. **12**(2): 1639-44.
289. Price, P.M., *Diabetes: Caught in the Akt?* Kidney Int, 2007. **71**(9): 839-841.
290. Bai, X., Li, X., Tian, J., Zhou, Z., *Antiangiogenic Treatment Diminishes Renal Injury and Dysfunction via Regulation of Local AKT in Early Experimental Diabetes.* PLoS One, 2014. **9**(4): e96117.
291. Chen, Z., Yuan, Y., Zou, X., Hong, M., Zhao, M., Zhao, Y., Liu, Y., Li, G., Zhu, Y., Luo, L., Bao, B., Bu, S., *Radix Puerariae and Fructus Crataegi mixture inhibits renal injury in type 2 diabetes via decreasing of AKT/PI3K.* BMC Complement Altern Med, 2017. **17**: 454.
292. Xu, F., Wang, Y., Cui, W., Yuan, H., Sun, J., Wu, M., Guo, Q., Kong, L., Wu, H., Miao, L., *Resveratrol Prevention of Diabetic Nephropathy Is Associated with the Suppression of Renal Inflammation and Mesangial Cell Proliferation: Possible Roles of Akt/NF-kappaB Pathway.* Int J Endocrinol, 2014. **2014**: 289327.
293. Lega, I.C., Wilton, A.S., Austin, P.C., Fischer, H.D., Johnson, J.A., Lipscombe, L.L., *The temporal relationship between diabetes and cancer: A population-based study.* Cancer, 2016. **122**(17): 2731-8.
294. Othman, E.M., Hintzsche, H., Stopper, H., *Signaling steps in the induction of genomic damage by insulin in colon and kidney cells.* Free Radic Biol Med, 2014. **68**: 247-57.
295. Habib, S.L., Liang, S., *Hyperactivation of Akt/mTOR and deficiency in tuberin increased the oxidative DNA damage in kidney cancer patients with diabetes.* Oncotarget, 2014. **5**(9): 2542-50.

296. Mollica, J.P., Oakhill, J.S., Lamb, G.D., Murphy, R.M., *Are genuine changes in protein expression being overlooked? Reassessing Western blotting.* Anal Biochem, 2009. **386**(2): 270-5.
297. Olive, D.M., *Quantitative methods for the analysis of protein phosphorylation in drug development.* Expert Rev Proteomics, 2004. **1**(3): 327-41.
298. Nomura, M., Takahashi, T., Nagata, N., Tsutsumi, K., Kobayashi, S., Akiba, T., Yokogawa, K., Moritani, S., Miyamoto, K., *Inhibitory mechanisms of flavonoids on insulin-stimulated glucose uptake in MC3T3-G2/PA6 adipose cells.* Biol Pharm Bull, 2008. **31**(7): 1403-9.
299. Erasalo, H., Hamalainen, M., Leppanen, T., Maki-Opas, I., Laavola, M., Haavikko, R., Yli-Kauhaluoma, J., *Natural Stilbenoids Have Anti-Inflammatory Properties in Vivo and Down-Regulate the Production of Inflammatory Mediators NO, IL6, and MCP1 Possibly in a PI3K/Akt-Dependent Manner.* J Nat Prod, 2018. **81**(5): 1131-1142.
300. Fruman, D.A., Chiu, H., Hopkins, B.D., Bagrodia, S., Cantley, L.C., Abraham, R.T., *The PI3K Pathway in Human Disease.* Cell, 2017. **170**(4): 605-635.
301. Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Remesy, C., Chap, H., Payrastre, B., *Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition.* Biochem Pharmacol, 1997. **53**(11): 1649-57.
302. Kong, D., Zhang, Y., Yamori, T., Duan, H., Jin, M., *Inhibitory activity of flavonoids against class I phosphatidylinositol 3-kinase isoforms.* Molecules, 2011. **16**(6): 5159-67.
303. Kang, Y., Kim, B.-G., Kim, S., Lee, Y., Yoon, Y., *Inhibitory potential of flavonoids on PtdIns(3,4,5)P3 binding with the phosphoinositide-dependent kinase 1 pleckstrin homology domain.* Bioorg Med Chem Lett, 2017. **27**(3): 420-426.
304. Ali, H.M., Abo-Shady, A., Sharaf Eldeen, H.A., Soror, H.A., Shousha, W.G., Abdel-Barry, O.A., Saleh, A.M., *Structural features, kinetics and SAR study of radical scavenging and antioxidant activities of phenolic and anilinic compounds.* Chem Cent J, 2013. **7**(1): 53.
305. Nile, S.H., Ko, E.Y., Kim, D.H., Keum, Y.-S., *Screening of ferulic acid related compounds as inhibitors of xanthine oxidase and cyclooxygenase-2 with anti-inflammatory activity.* Rev Bras Farmacogn, 2016. **26**(1): 50-55.
306. Ahmed, H., Moawad, A., Owis, A., AbouZid, S., Ahmed, O., *Flavonoids of Calligonum polygonoides and their cytotoxicity.* Pharm Biol, 2016. **54**(10): 2119-26.
307. Lahair, M.M., Howe, C.J., Rodriguez-Mora, O., McCubrey, J.A., Franklin, R.A., *Molecular pathways leading to oxidative stress-induced phosphorylation of Akt.* Antioxid Redox Signal, 2006. **8**(9-10): 1749-56.
308. Bors, W., Heller, W., Michel, C., Saran, M., *Flavonoids as antioxidants: determination of radical-scavenging efficiencies.* Methods Enzymol, 1990. **186**: 343-55.
309. Crasci, L., Lauro, M.R., *Natural antioxidant polyphenols on inflammation management: Anti-glycation activity vs metalloproteinases inhibition.* Crit Rev Food Sci Nutr, 2018. **58**(6): 893-904.
310. Amic, D., Davidovic-Amic, D., Beslo, D., Rastija, V., Lucic, B., Trinajstic, N., *SAR and QSAR of the antioxidant activity of flavonoids.* Curr Med Chem, 2007. **14**(7): 827-45.
311. *Molecular Operating Environment (MOE), 2013.08;*. 2018, Chemical Computing Group ULC: 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada,.
312. Labute, P., Williams, C., Feher, M., Sourial, E., Schmidt, J.M., *Flexible alignment of small molecules.* J Med Chem, 2001. **44**(10): 1483-90.
313. Chan, S.L., Labute, P., *Training a Scoring Function for the Alignment of Small Molecules.* J Chem Inf Model, 2010. **50**(9): 1724-35.
314. Enamine. *Enamine Store*. 2018 [cited 2018 24.07.]; Available from: https://www.enaminestore.com.
315. Life chemicals Inc. *Life chemicals - Synthesis and sales of novel organic compounds*. 2003 2018; Available from: http://www.lifechemicals.com/.
316. eMolecules Inc. *eMolecules: empowering drug discovery*. 2018 [cited 2018 24.07.]; Available from: https://www.emolecules.com/.
317. Novick, P.A., Ortiz, O.F., Poelman, J., Abdulhay, A.Y., Pande, V.S., *SWEETLEAD: an In Silico Database of Approved Drugs, Regulated Chemicals, and Herbal Isolates for Computer-Aided Drug Discovery.* PLoS One, 2013. **8**(11): e79568.
318. Wishart, D.S., Knox, C., Guo, A.C., Shrivastava, S., Hassanali, M., Stothard, P., Chang, Z., Woolsey, J., *DrugBank: a comprehensive resource for in silico drug discovery and exploration.* Nucleic Acids Res, 2006. **34**(Database issue): D668-D672.
319. *MOKA*. 2018, Molecular Discovery: Unit 501 Centennial Park, Centennial Avenue Elstree, WD6 3FG, Borehamwood, Hertfordshire, UNITED KINGDOM.
320. López-Nicolás, J.M., García-Carmona, F., *Aggregation State and pKa Values of (E)-Resveratrol As Determined by Fluorescence Spectroscopy and UV−Visible Absorption.* J Agric Food Chem, 2008. **56**(17): 7600-7605.

321. Amat, A., Clementi, C., De Angelis, F., Sgamellotti, A., Fantacci, S., *Absorption and Emission of the Apigenin and Luteolin Flavonoids: A TDDFT Investigation.* J Phys Chem A, 2009. **113**(52): 15118-15126.
322. Herrero-Martinez, J.M., Sanmartin, M., Roses, M., Bosch, E., Rafols, C., *Determination of dissociation constants of flavonoids by capillary electrophoresis.* Electrophoresis, 2005. **26**(10): 1886-95.
323. Milletti, F., Storchi, L., Sforna, G., Cruciani, G., *New and Original pKa Prediction Method Using Grid Molecular Interaction Fields.* J Chem Inf Model, 2007. **47**(6): 2172-2181.
324. Fröjdö, S., Cozzone, D., Vidal, H., Pirola, L., *Resveratrol is a class IA phosphoinositide 3-kinase inhibitor.* Biochem J, 2007. **406**(3): 511.
325. Kim, H.Y., Jung, S.K., Byun, S., Son, J.E., Oh, M.H., Lee, J., Kang, M.J., Heo, Y.S., Lee, K.W., Lee, H.J., *Raf and PI3K are the molecular targets for the anti-metastatic effect of luteolin.* Phytother Res, 2013. **27**(10): 1481-8.
326. Srinivasan, S., Jala, V., Honnenahally, K., Castellanos, J., Vermula, P.K., VanSaun, M., Merchant, N., Nagathihalli, N., *Abstract 5259: Urolithin A prevents pancreatic tumor growth and increases survival by inhibiting PI3K/PDK1 and STAT3 signaling.* Cancer Res, 2017. **77**(13 Supplement): 5259.
327. Walker, E.H., Pacold, M.E., Perisic, O., Stephens, L., Hawkins, P.T., Wymann, M.P., Williams, R.L., *Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine.* Mol Cell, 2000. **6**(4): 909-19.
328. Beno, B.R., Yeung, K.-S., Bartberger, M.D., Pennington, L.D., Meanwell, N.A., *A Survey of the Role of Noncovalent Sulfur Interactions in Drug Design.* J Med Chem, 2015. **58**(11): 4383-4438.
329. Valdes, A.M., Walter, J., Segal, E., Spector, T.D., *Role of the gut microbiota in nutrition and health.* BMJ, 2018. **361**: k2179.
330. Högger, P., *Nutrition-derived bioactive metabolites produced by gut microbiota and their potential impact on human health.* Nutr Med, 2013. **1**(1): 1.
331. Elena, R.M., Gabriela, G.D., Arnulfod, G.C., Enrique, C.A., *Studying the Gut Microbiome of Latin America and Hispanic/Latino populations. Insight into Obesity and Diabetes. Systematic Review.* Curr Diabetes Rev, 2018.
332. Kassaian, N., Feizi, A., Aminorroaya, A., Amini, M., *Probiotic and synbiotic supplementation could improve metabolic syndrome in prediabetic adults: A randomized controlled trial.* Diabetes Metab Syndr, 2018.
333. Puddu, A., Sanguineti, R., Montecucco, F., Viviani, G.L., *Evidence for the Gut Microbiota Short-Chain Fatty Acids as Key Pathophysiological Molecules Improving Diabetes.* Mediators Inflamm, 2014. **2014**: 9.
334. Li, D., Kirsop, J., Tang, W.H., *Listening to Our Gut: Contribution of Gut Microbiota and Cardiovascular Risk in Diabetes Pathogenesis.* Curr Diab Rep, 2015. **15**(9): 63.
335. Sun, Q., Wedick, N.M., Pan, A., Townsend, M.K., Cassidy, A., Franke, A.A., Rimm, E.B., Hu, F.B., van Dam, R.M., *Gut microbiota metabolites of dietary lignans and risk of type 2 diabetes: a prospective investigation in two cohorts of U.S. women.* Diabetes Care, 2014. **37**(5): 1287-95.
336. Duda-Chodak, A., Tarko, T., Satora, P., Sroka, P., *Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review.* Eur J Nutr, 2015. **54**(3): 325-41.
337. Seeram, N.P., Zhang, Y., McKeever, R., Henning, S.M., Lee, R.P., Suchard, M.A., Li, Z., Chen, S., Thames, G., Zerlin, A., Nguyen, M., Wang, D., Dreher, M., Heber, D., *Pomegranate juice and extracts provide similar levels of plasma and urinary ellagitannin metabolites in human subjects.* J Med Food, 2008. **11**(2): 390-4.
338. Kutschera, M., Engst, W., Blaut, M., Braune, A., *Isolation of catechin-converting human intestinal bacteria.* J Appl Microbiol, 2011. **111**(1): 165-75.
339. Saha, P., Yeoh, B.S., Singh, R., Chandrasekar, B., Vemula, P.K., Haribabu, B., Vijay-Kumar, M., Jala, V.R., *Gut Microbiota Conversion of Dietary Ellagic Acid into Bioactive Phytoceutical Urolithin A Inhibits Heme Peroxidases.* PLoS One, 2016. **11**(6): e0156811.
340. Larrosa, M., González-Sarrías, A., García-Conesa, M.T., Tomás-Barberán, F.A., Espín, J.C., *Urolithins, Ellagic Acid-Derived Metabolites Produced by Human Colonic Microflora, Exhibit Estrogenic and Antiestrogenic Activities.* J Agric Food Chem, 2006. **54**(5): 1611-1620.
341. Liberal, J., Carmo, A., Gomes, C., Cruz, M.T., Batista, M.T., *Urolithins impair cell proliferation, arrest the cell cycle and induce apoptosis in UMUC3 bladder cancer cells.* Invest New Drugs, 2017. **35**(6): 671-681.
342. Good, C.B., Pogach, L.M., *Should metformin be first-line therapy for patients with type 2 diabetes and chronic kidney disease? Informed patients should decide.* JAMA Intern Med, 2018. **178**(7): 911-912.
343. Driver, C., Bamitale, K.D.S., Kazi, A., Olla, M., Nyane, N.A., Owira, P.M.O., *Cardioprotective Effects of Metformin.* J Cardiovasc Pharmacol, 2018. **72**(2): 121-127.

344. van der Aa, M.P., Elst, M.A., van de Garde, E.M., van Mil, E.G., Knibbe, C.A., van der Vorst, M.M., *Long-term treatment with metformin in obese, insulin-resistant adolescents: results of a randomized double-blinded placebo-controlled trial.* Nutr Diabetes, 2016. **6**(8): e228.
345. Barzilai, N., Crandall, J.P., Kritchevsky, S.B., Espeland, M.A., *Metformin as a Tool to Target Aging.* Cell Metab, 2016. **23**(6): 1060-1065.
346. Chukwunonso Obi, B., Chinwuba Okoye, T., Okpashi, V.E., Nonye Igwe, C., Olisah Alumanah, E., *Comparative Study of the Antioxidant Effects of Metformin, Glibenclamide, and Repaglinide in Alloxan-Induced Diabetic Rats.* J Diabetes Res, 2016. **2016**: 1635361.
347. Hu, Y., Liu, J., Wang, G., Xu, Y., *The Effects of Exenatide and Metformin on Endothelial Function in Newly Diagnosed Type 2 Diabetes Mellitus Patients: A Case-Control Study.* Diabetes Ther, 2018. **9**(3): 1295-1305.
348. Aliper, A., Jellen, L., Cortese, F., Artemov, A., Karpinsky-Semper, D., Moskalev, A., Swick, A.G., Zhavoronkov, A., *Towards natural mimetics of metformin and rapamycin.* Aging (Albany NY), 2017. **9**(11): 2245-2268.
349. Lima, C.F., Azevedo, M.F., Araujo, R., Fernandes-Ferreira, M., Pereira-Wilson, C., *Metformin-like effect of Salvia officinalis (common sage): is it useful in diabetes prevention?* Br J Nutr, 2006. **96**(2): 326-33.
350. Algariri, K., Meng, K.Y., Atangwho, I.J., Asmawi, M.Z., Sadikun, A., Murugaiyah, V., Ismail, N., *Hypoglycemic and anti-hyperglycemic study of Gynura procumbens leaf extracts.* Asian Pac J Trop Biomed, 2013. **3**(5): 358-66.
351. Nyane, N.A., Tlaila, T.B., Malefane, T.G., Ndwandwe, D.E., Owira, P.M.O., *Metformin-like antidiabetic, cardio-protective and non-glycemic effects of naringenin: Molecular and pharmacological insights.* Eur J Pharmacol, 2017. **803**: 103-111.
352. Lee, S.H., Kwon, K.I., *Pharmacokinetic-pharmacodynamic modeling for the relationship between glucose-lowering effect and plasma concentration of metformin in volunteers.* Arch Pharm Res, 2004. **27**(7): 806-10.
353. Christensen, M.M., Brasch-Andersen, C., Green, H., Nielsen, F., Damkier, P., Beck-Nielsen, H., Brosen, K., *The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c.* Pharmacogenet Genomics, 2011. **21**(12): 837-50.
354. Isoda, K., Young, J.L., Zirlik, A., MacFarlane, L.A., Tsuboi, N., Gerdes, N., Schonbeck, U., Libby, P., *Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells.* Arterioscler Thromb Vasc Biol, 2006. **26**(3): 611-7.
355. He, L., Wondisford, Fredric E., *Metformin Action: Concentrations Matter.* Cell Metab, 2015. **21**(2): 159-162.
356. Liu, Y., Zhang, Y., Jia, K., Dong, Y., Ma, W., *Metformin inhibits the proliferation of A431 cells by modulating the PI3K/Akt signaling pathway.* Exp Ther Med, 2015. **9**(4): 1401-1406.
357. Li, Z., Wang, L., Luo, N., Zhao, Y., Li, J., Chen, Q., Tian, Y., *Metformin inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the phosphorylation of Akt.* Oncol Lett, 2018. **15**(5): 7948-7954.
358. Lalau, J.D., Lacroix, C., *Measurement of metformin concentration in erythrocytes: clinical implications.* Diabetes Obes Metab, 2003. **5**(2): 93-8.
359. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J., Moller, D.E., *Role of AMP-activated protein kinase in mechanism of metformin action.* J Clin Invest, 2001. **108**(8): 1167-1174.
360. Dhanya, R., Arya, A.D., Nisha, P., Jayamurthy, P., *Quercetin, a Lead Compound against Type 2 Diabetes Ameliorates Glucose Uptake via AMPK Pathway in Skeletal Muscle Cell Line.* Front Pharmacol, 2017. **8**: 336.
361. Cade, W.T., *Diabetes-Related Microvascular and Macrovascular Diseases in the Physical Therapy Setting.* Phys Ther, 2008. **88**(11): 1322-1335.
362. Jansson, P.A., *Endothelial dysfunction in insulin resistance and type 2 diabetes.* J Intern Med, 2007. **262**(2): 173-83.
363. Schalkwijk, C.G., Stehouwer, C.D., *Vascular complications in diabetes mellitus: the role of endothelial dysfunction.* Clin Sci (Lond), 2005. **109**(2): 143-59.
364. Lorenzi, M., Cagliero, E., Toledo, S., *Glucose toxicity for human endothelial cells in culture. Delayed replication, disturbed cell cycle, and accelerated death.* Diabetes, 1985. **34**(7): 621-7.
365. Ho, F.M., Lin, W.W., Chen, B.C., Chao, C.M., Yang, C.-R., Lin, L.Y., Lai, C.C., Liu, S.H., Liau, C.S., *High glucose-induced apoptosis in human vascular endothelial cells is mediated through NF-κB and c-Jun NH2-terminal kinase pathway and prevented by PI3K/Akt/eNOS pathway.* Cell Signal, 2006. **18**(3): 391-399.
366. Giardino, I., Edelstein, D., Brownlee, M., *BCL-2 expression or antioxidants prevent hyperglycemia-induced formation of intracellular advanced glycation endproducts in bovine endothelial cells.* J Clin Invest, 1996. **97**(6): 1422-8.

367. Varma, S., Lal, B.K., Zheng, R., Breslin, J.W., Saito, S., Pappas, P.J., Hobson, R.W., Durán, W.N., *Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction.* Am J Physiol Heart Circ Physiol, 2005. **289**(4): H1744-H1751.
368. Huang, Q., Sheibani, N., *High glucose promotes retinal endothelial cell migration through activation of Src, PI3K/Akt1/eNOS, and ERKs.* Am J Physiol Cell Physiol, 2008. **295**(6): C1647-C1657.
369. Bahadoran, Z., Mirmiran, P., Azizi, F., *Dietary polyphenols as potential nutraceuticals in management of diabetes: a review.* J Diabetes Metab Disord, 2013. **12**: 43-43.
370. Morikawa, T., Ninomiya, K., Akaki, J., Kakihara, N., Kuramoto, H., Matsumoto, Y., Hayakawa, T., Muraoka, O., Wang, L.B., Wu, L.J., Nakamura, S., Yoshikawa, M., Matsuda, H., *Dipeptidyl peptidase-IV inhibitory activity of dimeric dihydrochalcone glycosides from flowers of Helichrysum arenarium.* J Nat Med, 2015. **69**(4): 494-506.
371. Gonzalez-Abuin, N., Martinez-Micaelo, N., Blay, M., Pujadas, G., Garcia-Vallve, S., Pinent, M., Ardevol, A., *Grape seed-derived procyanidins decrease dipeptidyl-peptidase 4 activity and expression.* J Agric Food Chem, 2012. **60**(36): 9055-61.
372. Bar, J., Weber, A., Hoffmann, T., Stork, J., Wermann, M., Wagner, L., Aust, S., Gerhartz, B., Demuth, H.U., *Characterisation of human dipeptidyl peptidase IV expressed in Pichia pastoris. A structural and mechanistic comparison between the recombinant human and the purified porcine enzyme.* Biol Chem, 2003. **384**(12): 1553-63.
373. Cer, R.Z., Mudunuri, U., Stephens, R., Lebeda, F.J., *IC(50)-to-K(i): a web-based tool for converting IC(50) to K(i) values for inhibitors of enzyme activity and ligand binding.* Nucleic Acids Res, 2009. **37**(Web Server issue): W441-5.
374. Bakhtiarova, A., Taslimi, P., Elliman, S.J., Kosinski, P.A., Hubbard, B., Kavana, M., Kemp, D.M., *Resveratrol inhibits firefly luciferase.* Biochem Biophys Res Commun, 2006. **351**(2): 481-4.
375. Soares, S., Mateus, N., Freitas, V., *Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching.* J Agric Food Chem, 2007. **55**(16): 6726-35.
376. Röhrborn, D., Wronkowitz, N., Eckel, J., *DPP4 in Diabetes.* Front Immunol, 2015. **6**: 386.
377. Sell, H., Bluher, M., Kloting, N., Schlich, R., Willems, M., Ruppe, F., Knoefel, W.T., Dietrich, A., Fielding, B.A., Arner, P., Frayn, K.N., Eckel, J., *Adipose dipeptidyl peptidase-4 and obesity: correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro.* Diabetes Care, 2013. **36**(12): 4083-90.
378. Zilleßen, P., Celner, J., Kretschmann, A., Pfeifer, A., Racké, K., Mayer, P., *Metabolic role of dipeptidyl peptidase 4 (DPP4) in primary human (pre)adipocytes.* Sci Rep, 2016. **6**: 23074.
379. Das, S.S., Hayashi, H., Sato, T., Yamada, R., Hiratsuka, M., Hirasawa, N., *Regulation of dipeptidyl peptidase 4 production in adipocytes by glucose.* Diabetes Metab Syndr Obes, 2014. **7**: 185-94.
380. Gu, N., Tsuda, M., Matsunaga, T., Adachi, T., Yasuda, K., Ishihara, A., Tsuda, K., *Glucose regulation of dipeptidyl peptidase IV gene expression is mediated by hepatocyte nuclear factor-1alpha in epithelial intestinal cells.* Clin Exp Pharmacol Physiol, 2008. **35**(12): 1433-9.
381. Lee, S.A., Kim, Y.R., Yang, E.J., Kwon, E.J., Kim, S.H., Kang, S.H., Park, D.B., Oh, B.C., Kim, J., Heo, S.T., Koh, G., Lee, D.H., *CD26/DPP4 levels in peripheral blood and T cells in patients with type 2 diabetes mellitus.* J Clin Endocrinol Metab, 2013. **98**(6): 2553-61.
382. Post, S., van den Broek, A.J., Rensing, B.J., Pasterkamp, G., Goumans, M.J., Doevendans, P.A., *Reduced CD26 expression is associated with improved cardiac function after acute myocardial infarction: insights from the REPERATOR study.* J Mol Cell Cardiol, 2012. **53**(6): 899-905.
383. Pala, L., Mannucci, E., Pezzatini, A., Ciani, S., Sardi, J., Raimondi, L., Ognibene, A., Cappadona, A., Vannelli, B.G., Rotella, C.M., *Dipeptidyl peptidase-IV expression and activity in human glomerular endothelial cells.* Biochem Biophys Res Commun, 2003. **310**(1): 28-31.
384. Gum, J.R., Erickson, R.H., Hicks, J.W., Rius, J.L., Kim, Y.S., *Analysis of dipeptidyl peptidase IV gene regulation in transgenic mice: DNA elements sufficient for promoter activity in the kidney, but not the intestine, reside on the proximal portion of the gene 5'-flanking region.* FEBS Lett, 2000. **482**(1): 49-53.
385. Gu, N., Adachi, T., Matsunaga, T., Takeda, J., Tsujimoto, G., Ishihara, A., Yasuda, K., Tsuda, K., *Mutant HNF-1α and mutant HNF-1β identified in MODY3 and MODY5 downregulate DPP-IV gene expression in Caco-2 cells.* Biochem Biophys Res Commun, 2006. **346**(3): 1016-1023.
386. Bohm, S.K., Gum, J.R., Jr., Erickson, R.H., Hicks, J.W., Kim, Y.S., *Human dipeptidyl peptidase IV gene promoter: tissue-specific regulation from a TATA-less GC-rich sequence characteristic of a housekeeping gene promoter.* Biochem J, 1995. **311 (Pt 3)**: 835-43.
387. Chowdhury, H.H., Velebit, J., Radi, #x107, , N., #x161, Fran, #x10d, #x10d, , V., Kreft, M., Zorec, R., *Hypoxia Alters the Expression of Dipeptidyl Peptidase 4 and Induces Developmental Remodeling of Human Preadipocytes.* J Diabetes Res, 2016. **2016**: 9.

388. Bauvois, B., Djavaheri-Mergny, M., Rouillard, D., Dumont, J., Wietzerbin, J., *Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells.* Oncogene, 2000. **19**(2): 265-72.
389. Tan, E.Y., Mujoomdar, M., Blay, J., *Adenosine down-regulates the surface expression of dipeptidyl peptidase IV on HT-29 human colorectal carcinoma cells: implications for cancer cell behavior.* Am J Pathol, 2004. **165**(1): 319-30.
390. Tan, E.Y., Richard, C.L., Zhang, H., Hoskin, D.W., Blay, J., *Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing ERK1/2 activity via a novel pathway.* Am J Physiol Cell Physiol, 2006. **291**(3): C433-44.
391. Baricault, L., Fransen, J.A., Garcia, M., Sapin, C., Codogno, P., Ginsel, L.A., Trugnan, G., *Rapid sequestration of DPP IV/CD26 and other cell surface proteins in an autophagic-like compartment in Caco-2 cells treated with forskolin.* J Cell Sci, 1995. **108**(5): 2109.
392. Seamon, K.B., Padgett, W., Daly, J.W., *Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells.* Proc Natl Acad Sci U S A, 1981. **78**(6): 3363-7.
393. Uematsu, T., Tanaka, H., Yamaoka, M., Furusawa, K., *Effects of oral squamous cell carcinoma-derived TGF-beta1 on CD26/DPPIV expression in T cells.* Anticancer Res, 2004. **24**(2b): 619-24.
394. Real Hernandez, L.M., Fan, J., Johnson, M.H., Gonzalez de Mejia, E., *Berry Phenolic Compounds Increase Expression of Hepatocyte Nuclear Factor-1alpha (HNF-1alpha) in Caco-2 and Normal Colon Cells Due to High Affinities with Transcription and Dimerization Domains of HNF-1alpha.* PLoS One, 2015. **10**(9): e0138768.
395. Han, X., Shen, T., Lou, H., *Dietary Polyphenols and Their Biological Significance.* Int J Mol Sci, 2007. **8**(9): 950-988.
396. Johnson, M.H., de Mejia, E.G., *Phenolic Compounds from Fermented Berry Beverages Modulated Gene and Protein Expression To Increase Insulin Secretion from Pancreatic β-Cells in Vitro.* J Agric Food Chem, 2016. **64**(12): 2569-2581.
397. Peng, C.-H., Yang, Y.-S., Chan, K.-C., Wang, C.-J., Chen, M.-L., Huang, C.-N., *Hibiscus sabdariffa Polyphenols Alleviate Insulin Resistance and Renal Epithelial to Mesenchymal Transition: A Novel Action Mechanism Mediated by Type 4 Dipeptidyl Peptidase.* J Agric Food Chem, 2014. **62**(40): 9736-9743.
398. Huang, C.-N., Wang, C.-J., Yang, Y.-S., Lin, C.-L., Peng, C.-H., *Hibiscus sabdariffa polyphenols prevent palmitate-induced renal epithelial mesenchymal transition by alleviating dipeptidyl peptidase-4-mediated insulin resistance.* Food Funct, 2016. **7**(1): 475-482.
399. Cerda, B., Espin, J.C., Parra, S., Martinez, P., Tomas-Barberan, F.A., *The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans.* Eur J Nutr, 2004. **43**(4): 205-20.
400. Lefort, E.C., Blay, J., *The dietary flavonoid apigenin enhances the activities of the anti-metastatic protein CD26 on human colon carcinoma cells.* Clin Exp Metastasis, 2011. **28**(4): 337-49.
401. Moss, M.L., Lambert, M.H., *Shedding of membrane proteins by ADAM family proteases.* Essays Biochem, 2002. **38**: 141-53.
402. Rohrborn, D., Eckel, J., Sell, H., *Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells.* FEBS Lett, 2014. **588**(21): 3870-7.
403. Jessberger, S., Hogger, P., Genest, F., Salter, D.M., Seefried, L., *Cellular pharmacodynamic effects of Pycnogenol(R) in patients with severe osteoarthritis: a randomized controlled pilot study.* BMC Complement Altern Med, 2017. **17**(1): 537.
404. Ishibashi, Y., Matsui, T., Maeda, S., Higashimoto, Y., Yamagishi, S., *Advanced glycation end products evoke endothelial cell damage by stimulating soluble dipeptidyl peptidase-4 production and its interaction with mannose 6-phosphate/insulin-like growth factor II receptor.* Cardiovasc Diabetol, 2013. **12**: 125.
405. Uematsu, T., Urade, M., Yamaoka, M., *Decreased expression and release of dipeptidyl peptidase IV (CD26) in cultured peripheral blood T lymphocytes of oral cancer patients.* J Oral Pathol Med, 1998. **27**(3): 106-10.
406. Nargis, T., Chakrabarti, P., *Significance of circulatory DPP4 activity in metabolic diseases.* IUBMB Life, 2018. **70**(2): 112-119.
407. Nargis, T., Kumar, K., Ghosh, A.R., Sharma, A., Rudra, D., Sen, D., Chakrabarti, S., Mukhopadhyay, S., Ganguly, D., Chakrabarti, P., *KLK5 induces shedding of DPP4 from circulatory Th17 cells in type 2 diabetes.* Mol Metab, 2017. **6**(11): 1529-1539.
408. Stefanovic, V., Ardaillou, N., Vlahovic, P., Placier, S., Ronco, P., Ardaillou, R., *Interferon-gamma induces dipeptidylpeptidase IV expression in human glomerular epithelial cells.* Immunology, 1993. **80**(3): 465-70.

409. Jean Leandro dos, S., Chung Man, C., *Pan-Assay Interference Compounds (PAINS): Warning Signs in Biochemical-Pharmacological Evaluations.* Biochem Pharmacol, 2015. **4**(02): 1000e173.
410. Atanasov, A.G., Waltenberger, B., Pferschy-Wenzig, E.-M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E.H., Rollinger, J.M., Schuster, D., Breuss, J.M., Bochkov, V., Mihovilovic, M.D., Kopp, B., Bauer, R., Dirsch, V.M., Stuppner, H., *Discovery and resupply of pharmacologically active plant-derived natural products: A review.* Biotechnol Adv, 2015. **33**(8): 1582-1614.
411. Otagiri, M., Otagiri, Y., Perrin, J.H., *Some fluorescent investigations of the interaction between the enantiomers of warfarin and human serum albumin.* Int J Pharm, 1979. **2**(5): 283-294.
412. Gul, S., Gribbon, P., *Exemplification of the challenges associated with utilising fluorescence intensity based assays in discovery.* Expert Opin Drug Discov, 2010. **5**(7): 681-90.
413. Zou, L., Harkey, M.R., Henderson, G.L., *Effects of intrinsic fluorescence and quenching on fluorescence-based screening of natural products.* Phytomedicine, 2002. **9**(3): 263-7.
414. Les, F., Arbones-Mainar, J.M., Valero, M.S., Lopez, V., *Pomegranate polyphenols and urolithin A inhibit alpha-glucosidase, dipeptidyl peptidase-4, lipase, triglyceride accumulation and adipogenesis related genes in 3T3-L1 adipocyte-like cells.* J Ethnopharmacol, 2018.
415. Elisakit.com Pty Ltd., A. *ELISA Analysis*. 2012 [cited 2015, 30.09.]; Available from: http://elisaanalysis.com/.
416. Wewer Albrechtsen, N.J., Bak, M.J., Hartmann, B., Christensen, L.W., Kuhre, R.E., Deacon, C.F., Holst, J.J., *Stability of glucagon-like peptide 1 and glucagon in human plasma.* Endocr Connect, 2015. **4**(1): 50-7.
417. DeChancie, J., Houk, K.N., *The origins of femtomolar protein-ligand binding: hydrogen-bond cooperativity and desolvation energetics in the biotin-(strept)avidin binding site.* J Am Chem Soc, 2007. **129**(17): 5419-29.
418. Fujiwara, K., Tsuru, D., *New chromogenic and fluorogenic substrates for pyrrolidonyl peptidase.* J Biochem, 1978. **83**: 1145-1149.
419. Shapiro, S.S., Wilk, M.B., *An analysis of variance test for normality (complete samples)†.* Biometrika, 1965. **52**(3-4): 591-611.
420. Zaiontz, C. *Real Statistics Using Excel*. 2016 [cited 2016, 26.03.]; Available from: http://www.real-statistics.com/tests-normality-and-symmetry/statistical-tests-normality-symmetry/shapiro-wilk-test/.
421. Vasavada, N. *Online Web Statistical Calculators* 2016 [cited 2018, 26.03.]; Available from: http://astatsa.com/OneWay_Anova_with_TukeyHSD/.
422. Edgell, C.J., McDonald, C.C., Graham, J.B., *Permanent cell line expressing human factor VIII-related antigen established by hybridization.* Proc Natl Acad Sci U S A, 1983. **80**(12): 3734-7.
423. Berthois, Y., Katzenellenbogen, J.A., Katzenellenbogen, B.S., *Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture.* Proc Natl Acad Sci U S A, 1986. **83**(8): 2496-500.
424. Glover, J.F., Irwin, J.T., Darbre, P.D., *Interaction of phenol red with estrogenic and antiestrogenic action on growth of human breast cancer cells ZR-75-1 and T-47-D.* Cancer Res, 1988. **48**(13): 3693-7.
425. Uhlenhut, K., Hogger, P., *Pitfalls and limitations in using 4,5-diaminofluorescein for evaluating the influence of polyphenols on nitric oxide release from endothelial cells.* Free Radic Biol Med, 2012. **52**(11-12): 2266-75.
426. Chen, Z.P., Mitchelhill, K.I., Michell, B.J., *AMP-activated protein kinase phosphorylation of endothelial NO synthase.* FEBS Lett, 1999. **443**(3): 285-289.
427. Montagnani, M., Chen, H., Barr, V.A., Quon, M.J., *Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179).* J Biol Chem, 2001. **276**(32): 30392-8
428. Nim, T.H., Luo, L., White, J.K., Clément, M.-V., Tucker-Kellogg, L., *Non-canonical Activation of Akt in Serum-Stimulated Fibroblasts, Revealed by Comparative Modeling of Pathway Dynamics.* PLoS Comput Biol, 2015. **11**(11): e1004505.
429. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): 265-75.
430. Burnette, W.N., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.* Anal Biochem, 1981. **112**(2): 195-203.
431. Taylor, S.C., Posch, A., *The Design of a Quantitative Western Blot Experiment.* Biomed Res Int, 2014. **2014**: 8.
432. *ImageJ, Image Processing and Analysis in Java*. 2017 [cited 2017, 22.06.]; Available from: https://imagej.nih.gov/ij/download.html.
433. Todaro, G.J., Green, H., *Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines.* J Cell Biol, 1963. **17**: 299-313.

434. Green, H., Meuth, M., *An established pre-adipose cell line and its differentiation in culture.* Cell, 1974. **3**(2): 127-133.
435. Zebisch, K., Voigt, V., Wabitsch, M., Brandsch, M., *Protocol for effective differentiation of 3T3-L1 cells to adipocytes.* Anal Biochem, 2012. **425**(1): 88-90.
436. Public Health England, U.K. *ECACC, Health Protection Agency culture collections*. 2018 [cited 2018, 28.05.]; Available from: http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=86052701&collection=ecacc_gc.
437. Mehra, A., Macdonald, I., Pillay, T.S., *Variability in 3T3-L1 adipocyte differentiation depending on cell culture dish.* Anal Biochem, 2007. **362**(2): 281-3.
438. Sheng, X., Tucci, J., Malvar, J., Mittelman, S.D., *Adipocyte differentiation is affected by media height above the cell layer.* Int J Obes (Lond), 2014. **38**(2): 315-20.
439. Sato, T., Kotake, D., Hiratsuka, M., Hirasawa, N., *Enhancement of inflammatory protein expression and nuclear factor Kappab (NF-Kappab) activity by trichostatin A (TSA) in OP9 preadipocytes.* PLoS One, 2013. **8**(3): e59702.