THE INFLUENCE OF ANTHOCYANIN TREATMENT ON THE MESENTERIC ADIPOSE TISSUE EXPRESSION OF eNAMPT IN ANIMAL MODELS OF OBESITY

MARIA MARGARIDA NOBRE PROJECTO

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Human Nutrition and Metabolism
Dissertação para obtenção do grau de Mestre em Nutrição Humana e Metabolismo

at Faculdade de Ciências Médicas | NOVA Medical School of Universidade NOVA de Lisboa

September, 2022

THE INFLUENCE OF ANTHOCYANIN TREATMENT ON THE MESENTERIC ADIPOSE TISSUE EXPRESSION OF eNAMPT IN ANIMAL MODELS OF OBESITY

Maria Margarida Nobre Projecto

Supervisors: Marta P Silvestre, Invited Assistant Professor at Faculdade de Ciências Médicas | NOVA Medical School of Universidade NOVA de Lisboa
Ana Faria, Assistant Professor at Faculdade de Ciências Médicas | NOVA Medical School of Universidade NOVA de Lisboa

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Human Nutrition and Metabolism

September, 2022

This study was supported by National Funds through FCT – Fundação para a Ciência e a Tecnologia (FCT) within the project PTDC/BAA-AGR/7419/2020.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors, Professora Doutora Marta Silvestre, and Professora Doutora Ana Faria, for their professionalism, guidance, and availability. Sharing their knowledge and vast experience was a great asset to me and the elaboration of this thesis.

I also want to thank and acknowledge the entire faculty members of this Master for the quality of teaching and the encouragement of scientific investigation, right from the beginning. In particular, to Professora Doutora Diana Teixeira and Professor Doutor João Araújo for the essential support provided in this work.

Just as important is my gratitude to Pedro, Shámila, and especially Maria João for the fundamental support, collaboration, and company during the laboratory experience.

Finally, a big thank you to my family for their support and words of encouragement throughout my academic journey, which allowed me to get here today.

ABBREVIATIONS

AT	Adipose Tissue
ANKO	Adipose Tissue-Specific Nampt Knockout
AUC	Area Under the Curve
BAT	Brown Adipose Tissue
BE	Standard Diet + Blackberry Extract
BMI	Body Mass Index
C	Standard Diet
Cy-3-G	Cyanidin-3-O-β-Glucoside
eNAMPT	Extracellular Nicotinamide Phosphoribosyltransferase
FRD	Fructose Rich Diet
GSIS	Glucose-Stimulated Insulin Secretion
GTT	Glucose Tolerance Test
HFD	High-Fat Diet
HFDBE	High-Fat Diet + Blackberry Extract
HRP	Horse-Radish Peroxidase
iNAMPT	Intracellular Nicotinamide Phosphoribosyltransferase
IL-6	Interleukin 6
IR	Insulin Resistance
IS	Insulin Sensitivity
ITT	Insulin Tolerance Test
mAT	Mesenteric Adipose Tissue
NAD	Nicotinamide Adenine Dinucleotide
NAMPT	Nicotinamide Phosphoribosyltransferase
NAMPT +/–	NAMPT-deficient Heterozygous
NF-KB	Nuclear Factor Kappa B
NMN	Nicotinamide Mononucleotide
NMNAT	Nicotinic Acid Mononucleotide Adenylyltransferase
NMS|FCM	NOVA Medical School | Faculdade de Ciências Médicas
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene difluoride

RIPA	Radio-Immunoprecipitation
scAT	Subcutaneous Adipose Tissue
SD	Standard Deviation
SEM	Standard Error of The Mean
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
STAT3	Signal Transducer and Activator of Transcription 3
T2D	Type 2 Diabetes
TPBS	PBS containing 0,1% (vol/vol) Tween-20
TLR4	Toll-like Receptor 4
WAT	White Adipose Tissue
WB	Western Blot

ABSTRACT

Introduction: Nicotinamide Phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme for NAD biosynthesis. Its extracellular form (eNAMPT), mainly secreted by visceral fat, has been shown to have bimodal, concentration- and structure-functional-dependent effects in important metabolic pathways and has been connected to a wide variety of diseases. Data suggests that as serum eNAMPT concentration rises to pathophysiological levels, as in obesity and type 2 diabetes (T2D), eNAMPT adopts a monomeric form capable of proinflammatory NAD-independent effects. Strategies to block the actions of the eNAMPT monomer could represent promising therapeutic approaches for obesity-related metabolic disorders. Consumption of anthocyanin rich foods appears to prevent or treat obesity-related consequences, such as T2D, inflammation and oxidative stress, but the mechanism behind this is unknown. Moreover, anthocyanins have been shown to inhibit the secretion of eNAMPT in animal models. With this study, we aim to understand if treating a rat model of obesity with anthocyanins could abrogate the impact of a high fat diet in the expression of monomeric eNAMPT.

Methods: Mesenteric adipose tissue (mAT) was obtained from four groups of male Wistar rats, treated with different diets: (C) standard diet; (BE) standard diet + blackberry extract; (HFD) high-fat diet; (HFDBE) high-fat diet + blackberry extract. eNAMPT monomer's protein expression was measured by Western Blotting, after protein extraction and quantification from mAT, to access the differences between the animals fed a standard diet and those of increased metabolic risk – HFD, with and without treatment with anthocyanins.

Results: The mAT from HFD rats displayed a higher expression of eNAMPT monomer, compared to C rats (138,6% ± 11,4% HFBE vs 100% C, p=0,01). The anthocyanin's treatment influence on mAT eNAMPT monomer expression was also assessed. mAT eNAMPT monomer expression was significantly decreased in the HFDBE group compared to HFD (-54,1 ± 15,3 % [-89.4, -18.8], p<0,01).

Conclusions: Anthocyanin consumption might be an interesting dietary approach to abrogate the impact of a high fat diet on the rise of monomeric eNAMPT in mesenteric adipose tissue. Ultimately, our results suggest that long-term anthocyanin treatment/supplementation might be effective for sustaining lower levels of monomeric eNAMPT in the context of diet-induced obesity, potentially preventing or delaying the consequent metabolic impairments such as the alarming epidemic of T2D.

Keywords: Adipose tissue, Anthocyanins, eNAMPT, Nicotinamide Phosphoribosyltransferase, Obesity, Type 2 Diabetes

RESUMO

Introdução: A Nicotinamida Fosforibosiltranferase (NAMPT) é a enzima limitante no processo de biossíntese de NAD. A sua forma extracelular (eNAMPT), secretada principalmente pelo tecido adiposo visceral, demonstrou ter efeitos bimodais, dependentes da concentração e da estrutura funcional, em importantes vias metabólicas e tem sido associada a uma ampla variedade de doenças. Dados sugerem que à medida que a concentração sérica de eNAMPT aumenta para níveis fisiopatológicos, como na obesidade e diabetes tipo 2 (T2D), a eNAMPT adota uma forma monomérica capaz de efeitos pró-inflamatórios independentes do NAD. Possíveis estratégias que bloqueiem as ações do eNAMPT monomérico podem representar abordagens terapêuticas promissoras para os distúrbios metabólicos relacionados com a obesidade. O consumo de alimentos ricos em antocianinas parece prevenir ou tratar consequências relacionadas com a obesidade, como T2D, inflamação e stress oxidativo, mas o mecanismo subjacente é ainda desconhecido. Para além disso, as antocianinas demonstraram inibir a secreção de eNAMPT em modelos animais. Com este estudo, pretendemos compreender se o tratamento de um modelo animal de obesidade com antocianinas poderia anular o impacto de uma dieta rica em gordura na expressão de eNAMPT monomérico.

Métodos: O tecido adiposo mesentérico (mAT) foi obtido a partir de quatro grupos de ratos Wistar machos, tratados com diferentes dietas: (C) dieta standard; (BE) dieta standard + extrato de amora; (HFD) dieta rica em gordura; (HFDBE) dieta rica em gordura + extrato de amora. A expressão proteica do eNAMPT monomérico foi medida por Western Blotting, após a extração e quantificação das proteínas do mAT, para avaliar as diferenças entre os animais alimentados com dieta standard e os que apresentam risco metabólico aumentado – HFD, com e sem tratamento com antocianinas.

Resultados: No mAT de ratos HFD, a expressão do monómero eNAMPT foi 38,6% mais elevada quando comparado com C (138,6% ± 11,4% HFBE vs 100% C, p=0,01). A influência do tratamento com antocianinas na expressão do eNAMPT monomérico no mAT também foi avaliada em ratos alimentados com HFD e C. O eNAMPT monomérico diminuiu significativamente em ratos alimentados com HFDBE em comparação com HFD (-54,1 ± 15,3 % [-89.4, -18.8], p<0,01).

Conclusão: O consumo de antocianinas parece ser uma abordagem dietética interessante para anular o impacto de uma dieta rica em gordura no aumento de eNAMPT monomérico no tecido adiposo mesentérico. Em última análise, os nossos resultados sugerem que o tratamento/suplementação com antocianinas, a longo prazo, pode ser eficaz para sustentar níveis mais baixos de eNAMPT monomérico no contexto de uma obesidade induzida pela dieta, contribuindo para combater outras alterações metabólicas, como a alarmante epidemia de T2D.

Palavras-chave: Antocianinas, Diabetes tipo 2, eNAMPT, Nicotinamida Fosforibosiltranferase, Obesidade, Tecido Adiposo

CONTENTS

TABLE LIST

FIGURE LIST

1. INTRODUCTION

1.1 Adipose Tissue and Adipokines

Adipose tissue (AT) is the most effective energy-storing organ in the body, having a major role in whole-body energy homeostasis (1,2). If, for a long time, AT was considered an organ whose only function was to store energy in the form of fat, nowadays it is studied with great interest, being considered an endocrine organ, for producing and secreting a variety of endocrine and paracrine factors, namely adipokines, and inflammatory mediators (1–6).

AT comprises white (WAT) and brown (BAT) adipose tissue. BAT is essential for thermoregulatory thermogenesis in mammals, through efficient heat production. Mature brown adipocytes have abundant mitochondria, and their thermogenic capacity is also important for body weight regulation, by promoting energy expenditure (7,8). WAT is mainly composed of adipocytes, where triglycerides are deposited, and from where they can be mobilized as an energy source when needed. It also comprises pre-adipocytes, fibroblasts, and endothelial and immune cells, such as macrophages (9).

In WAT, the adipokines are secreted mainly by pre-adipocytes and mature adipocytes. These small hormonal factors are key regulators of various cellular biological and physiological processes, being involved in the management of nutrient intake through appetite regulation, immune and inflammatory aspects, insulin sensitivity (IS) and insulin resistance (IR), vascular homeostasis and in energy expenditure, and lipid and glucose metabolism (3,4). As so, they have a critical role in maintaining metabolic homeostasis, by creating a crosstalk between AT and other major metabolic organs, specifically the liver, pancreas, and muscle, as well as the central nervous system. Excessive adiposity and/or adipocyte dysfunction may lead to changes in adipokines' concentrations and patterns, and modification of their pathways, resulting in defective organ communications and therefore, metabolic irregularities in those tissues. Consequently, adipokines are critical pathological components in the development of metabolic diseases associated with obesity as well as clinically relevant markers of AT function (2,5).

WAT is also classified according to the regional distribution throughout the body as subcutaneous adipose tissue (scAT) when located under the skin, and visceral adipose tissue (vAT) when accumulated in visceral/intra-abdominal depots (10,11). This distribution is an important predictor for the development of metabolic dysfunction associated with obesity. vAT is characterized by a greater presence of macrophages and a more complex and exacerbated inflammatory response, in the context of obesity, compared to scAT. vAT inflammation is associated with development of obesity-related metabolic disorders, such as systemic insulin resistance and type 2 diabetes (T2D) (10,11). These visceral depots in humans include the omental, mesenteric, retroperitoneal, gonadal, and pericardial adipose tissues. Mesenteric adipose tissue (mAT) is located in the double fold of peritoneum and is in contact with intestines at the site of mesentery attachment (12).

1.2 Nicotinamide Phosphoribosyltransferase (NAMPT)

The nicotinamide phosphoribosyltransferase (NAMPT) is a pleiotropic protein that exists in an intracellular and extracellular form. Intracellular NAMPT (iNAMPT), is well known as an essential enzyme that acts as a dimeric type II phosphoribosyltransferase in the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway, by catalysing the production of nicotinamide mononucleotide (NMN) (13). NMN is then converted to NAD by another enzyme - nicotinamide acid mononucleotide adenylyltransferase (NMNAT) (14,15) (Figure 1).

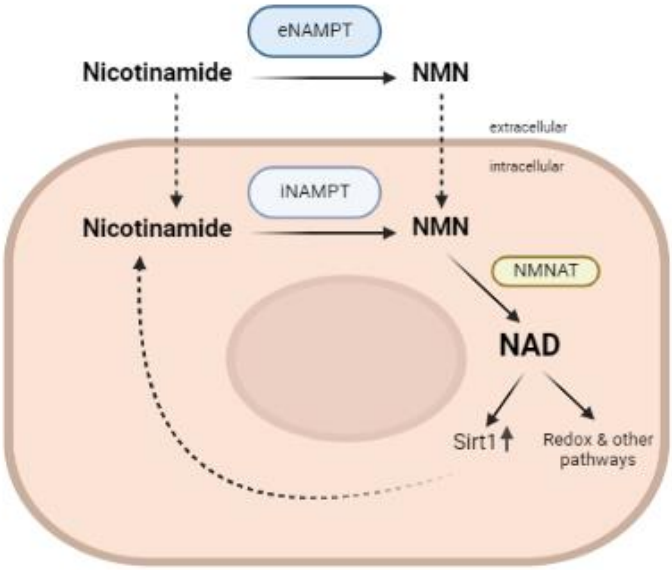


Figure 1 - NAD biosynthesis pathway from nicotinamide. The rate-limiting step in mammalian NAD biosynthesis from nicotinamide is catalysed by nicotinamide phosphoribosyltransferase (NAMPT) to produce nicotinamide mononucleotide (NMN), which is then converted to nicotinamide adenine dinucleotide (NAD) by nicotinamide mononucleotide adenylyltransferase (NMNAT). NAMPT exists as an intracellular and extracellular form, as so its biosynthetic activity occurs in both conditions. Adapted from Revollo et al., 2007.

NAD is a central coenzyme in cell metabolism, participating in redox reactions - essential in all living cells. It acts as an electron-accepting and donating agent, particularly in cellular respiration and oxidative phosphorylation, enabling the body to obtain energy from nutrients (16). NAD and its derivatives have also been acknowledged as important communicating extracellular molecules in some signalling pathways and are recognized as important factors in transcriptional regulation mechanisms (14,15). Although NAMPT-mediated NAD biosynthesis is the predominant pathway in mammals, NAD can also be generated by precursors other than nicotinamide, such as tryptophan, nicotinic acid, and nicotinamide riboside (14,16).

When surveying the tissue distribution of iNAMPT in mice, Revollo et al. demonstrated that iNAMPT protein expression levels were the highest in BAT, liver, and kidney. Heart, WAT, lung, spleen, testis, and skeletal muscle showed intermediate to very low levels of iNAMPT, while in the brain and pancreas it was undetectable. These findings suggest that iNAMPT-mediated NAD biosynthesis might significantly differ amidst different tissues (15).

The extracellular form of NAMPT (eNAMPT), also known as pre-B cell colony enhancing factor or visfatin, exhibits robust, even higher NAD biosynthetic activity than iNAMPT (15,17). The exact mechanism of eNAMPT's secretion is still under investigation, but it seems to be secreted mainly by adipocytes, through a non-classical pathway (15,18), being lately one of the adipokines with greater research interest.

The physiological role of eNAMPT, other than the enzymatic one, has been a matter of much debate. It has been shown that eNAMPT leads to different biological reactions by diverse types of cells, possibly through the activation of intracellular signalling pathways (19). Results from different studies have been controversial and the mechanisms by which eNAMPT exerts proinflammatory properties or is protective against cellular stress or toxicity is still subject of study. Indeed, the relationship between NAMPT and cellular homeostasis is highly complex (19). For instance, it has been shown that eNAMPT binds to Toll-like receptor 4 (TLR4) and activates the nuclear factor kappa B (NF-KB) signalling pathway (20,21). This causes the expression of various markers of inflammation and oxidative stress, as well as cell survival and death pathways regulation (22,23). In fact, there are several reports of eNAMPT having proinflammatory and immunomodulating properties. After murine eNAMPT administration in mice, circulating levels of interleukin 6 (IL-6), a cytokine that is strongly implicated in inflammatory and immunological processes, arose (3). Some authors observed that recombinant eNAMPT dose-dependently induced mRNA expression levels of cytokines such as IL-1, IL-6, IL-10, and TNF-α and provoked the production of these cytokines in human lymphocytes and monocytes, in which IL-6 up-regulation and production was the most pronounced (3,21).

For the first time in 2008, investigators found that eNAMPT could promote cell survival in macrophages, by inhibiting endoplasmic reticulum stress-induced apoptosis. This mechanism occurs by activating an IL-6/Signal Transducer and Activator of Transcription 3 (STAT3) signalling pathway (24). Macrophages have an important and preponderant role in the pathophysiology of comorbidities associated with obesity, such as atherosclerosis (25), inflammation (26), and cancer (27). Therefore, something that changes the dynamics of survival-apoptosis of these immune cells will have an impact on the emergence of diseases related to obesity. Prolonged macrophage survival was suggested to prop up the inflammatory response, known as a crucial factor underlying obesity-related metabolic diseases (24). Interestingly these findings were not dependent on the enzymatic activity of eNAMPT, corroborated by the fact that STAT3 phosphorylation was not induced by NMN – NAMPT's enzymatic product - and because the

inhibitor of NAMPT's enzymatic activity - FK866 - also did not prevent eNAMPT's ability to stimulate phosphorylation of STAT3 (24). As Y. Li et al. also report, it seems questionable that cells release an enzyme that is capable of activating certain metabolic pathways in a non-enzymatic way. Later, a possible justification for this, related to structural and functional changes of the eNAMPT protein, will be addressed.

1.3 NAMPT In The Context of Metabolic Disease – Obesity and Diabetes

eNAMPT has recently been shown to have an important connection to a wide variety of diseases. There is in fact evidence showing enhanced mRNA expression of NAMPT and higher circulating levels of eNAMPT in obesity, T2D, cancer, and other inflammatory conditions (18,28,29), including inflammatory bowel diseases ((3,30)) rheumatoid arthritis (31), atherosclerosis, myocardial infarction, and severe generalized psoriasis (3,13,30).

The biological action of NAMPT in obesity and obesity-related disorders, such as T2D, has been interestingly studied since Fukuhara et al. demonstrated that eNAMPT exerted a glucose-lowering effect in a diabetic mouse model. However, the article ended up retracted due to the controversy regarding the insulin receptor-binding activity of eNAMPT that could not be reproduced (32). Despite the significant interest, there is a lot of controversy over the association between circulating levels of eNAMPT and obesity-related parameters, and the effects of eNAMPT in the context of T2D, particularly regarding the role of this protein in beta cell function.

The results from a meta-analysis of 13 observational studies involving 644 subjects showed a significant increase in eNAMPT concentration in participants with overweight/obesity compared to normal body mass index (BMI) controls (28)). Moreover, Fukuhara et al. demonstrated that eNAMPT was more abundantly secreted by vAT in both humans and mice, than in scAT (13,32). As mentioned, the article was retracted due to the lack of reproducibility of the main result – eNAMPT insulin-mimetic effect – however other authors corroborated the significant association between eNAMPT levels and visceral fat (19,33,34).

To investigate the function of eNAMPT specifically secreted and regulated by AT, Yoon et al. created and analysed adipose tissue-specific Nampt knockout (ANKO) mice, which showed reductions in plasma eNAMPT levels and NAD levels, resulting also in severe impairments in IS and glucose-stimulated insulin secretion (GSIS) (35). In line with this knowledge, AT may be fundamental to regulate beta cell function through secretion of eNAMPT and consequent extracellular biosynthesis of NMN, as fully differentiated adipocytes are a natural producer of eNAMPT (13,15,35). Revollo et al. showed that NAMPT-deficient heterozygous (NAMPT +/–) mice had a defect in NAD biosynthesis and in GSIS in pancreatic islets. Administration of NMN could completely ameliorate these defects, strongly indicating that they were due to a lack of NAMPT-mediated NAD biosynthesis (15). Adipocyte dysfunction, with ultimate dysregulation of adipokines, particularly eNAMPT, can lead to alterations in IS and the development of T2D, with simultaneously worsening of inflammation and obesity (20,36,37). Consistent evidence shows

that T2D patients present higher levels of eNAMPT compared to healthy controls (36–38)). In a 2011 meta-analysis, this evidence was reinforced as it was shown that eNAMPT was significantly increased in subjects diagnosed with T2D (28). This relationship between NAMPT activity and T2D appears to be related to pancreatic tissue being more susceptible to changes in NAMPT-mediated NAD biosynthesis, compared to other tissues. Because of the almost total absence of iNAMPT, islets rely on extracellular sources of NAD intermediates, such as NMN, to maintain their NAD levels adequate for their functions (14,15). Moreover, available evidence suggests that the elevated eNAMPT concentration in T2D may be a physiologic protective response to a hyperglycemic environment (28). Although, at supraphysiological levels, the effect apparently turns deleterious (20,39).

As discussed earlier, according to some data, the inhibitor of NAMPT's activity - FK866 - has been shown to be ineffective, indicating that at least part of NAMPT's effects is independent of its enzymatic activity ((18,24)). Dimerization of eNAMPT is essential for its enzymatic function, and at physiological levels, in healthy individuals, eNAMPT is found mainly in the dimeric form (~100kDa). Yet, Li et al. showed that NAMPT mutants, which were unable to dimerize, maintained a cytokine-like activity (24). For example, as discussed previously, the anti-apoptotic effect of NAMPT does not require dimerization, suggesting that it is the monomeric form (~50kDa) that maintains this pro-inflammatory effect (13).

A study deepened the characterization of this structure-functional difference of eNAMPT in the deterioration of pancreatic beta cell function and its relationship with T2D, in order to understand the contradictory effects of eNAMPT on beta cell function (protective or deleterious), previously described in the literature (40). The study concluded that concentration played an important role in these changes in structure and function, and that dimeric eNAMPT, found at low physiological levels promotes NAD biosynthesis, while at a supraphysiological state eNAMPT adopted a monomeric form capable of pro-inflammatory mechanisms, independent of NAD, deleterious for beta cell function (40).

1.4 Anthocyanins - Health Promotion and Potential Effect on eNAMPT in Context of Metabolic Disease

Despite obesity being the main risk factor for T2D (37,41), diet is an unquestionable modifiable factor that may influence the risk of both obesity and diabetes (42). A high fat diet in the context of overnutrition leads to increased lipid deposition in both adipose and non-adipose tissues (43,44). In vivo studies have shown that animals fed high-fat diets develop obesity and diabetes, within weeks (45–47)). Furthermore, a high fat diet has been shown to induce alterations in blood lipid profile and in circulating proinflammatory cytokines (48,49). In contrast to the typical "western diet", high in sugar and fat, a fruit and vegetables rich diet has been associated with a lower risk of obesity and related co-morbidities (50,51). In particular, observational studies show that a higher intake of fruits and vegetables has been inversely associated with the risk of T2D

(52–55). However, the number of studies is limited and findings regarding these associations have not been consistent (56–58).

In recent years, berries have been a field of research that has grown considerable interest, due to their potential, as a natural product, for health promotion and disease prevention (59–61). Berries have been associated with cognitive function and anti-inflammatory and antioxidant responses (62,63), as well as with the reduction of adipocyte death and lower adipocyte size in mAT (46,61). In addition, a growing body of evidence, including prospective cohort studies, suggests that higher intakes of berries are inversely associated with T2D risk (52–54). Considering this, in other findings, including those from clinical trials, berries have shown an improvement in glucose response and in IS (52–54). These beneficial outcomes of fruit, in particular berries, seem to be connected to their composition in terms of dietary fiber and polyphenols (64,65). Dietary phytochemicals (such as polyphenols) may result in synergistic activity that can offer advantages over treatments with single chemicals that may comprehend adverse side effects (59,60).

Anthocyanins are a group of polyphenolic natural pigments that belong to a broad class of secondary metabolites called flavonoids, abundant in various brightly colored fruit and vegetables (59–61). Despite the limited number of clinical studies, several in vitro and in vivo studies confirmed that the consumption of anthocyanins rich food prevents or treats obesity-related consequences like T2D, inflammation and oxidative stress. (66,67). The studies that have been conducted to evaluate the biological functions of dietary anthocyanins on inflammation and obesity have shown that their anti-obese properties rely on their ability to control food consumption and energy metabolism and improve inflammatory response. However, these favorable effects were dependent on several physiological, immunological, neurological, and metabolic interconnected events (66,67). Dietary anthocyanins also appear to ameliorate IR (42,46,61,64,65,68). Experimental studies have demonstrated that the benefits in T2D associated with anthocyanins may be related to improvements in IS (60), through diverse mechanisms ranging from inhibiting carbohydrate digestion in the gut, pancreatic beta cell protection, and insulin secretion to enhancing IS in vital organs (59). Cyanidin-3-O-β-glucoside (Cy-3-G), an abundant anthocyanin, was able to regulate the expression of adipokines in BAT, which were disrupted by a high fat diet supplemented with cholesterol (8). Furthermore, supplementation with purified anthocyanins decreased eNAMPT in patients with prediabetes or newly diagnosed diabetes and showed improvements in glucolipid metabolic parameters (69).

Taken together, these studies suggest a beneficial effect of anthocyanins as preventive strategy for obesity–related metabolic abnormalities, potentially through a pathway involving NAMPT. However, the mechanism by which anthocyanins play a role in obesity through eNAMPT has not been elucidated nor was the relationship between anthocyanin treatment and the specific concentration of eNAMPT in its dimeric/monomeric form.

2. AIMS

This study seeks to help closing the gap in current literature, by investigating the differences in eNAMPT's expression, in its monomeric form, in the mesenteric adipose tissue of animal models in the context of diet-induced obesity and increased metabolic risk (fed a high fat diet). Moreover, the study aims to understand how the effect of such obesogenic diet in the expression of monomeric eNAMPT is impacted by an anthocyanin treatment. Ultimately, the study aims to give us an insight into early mechanisms of disease prevention, potentially contributing to the therapeutic strategies for metabolic disorders.

3. METHODOLOGY

3.1 Experimental Design

This study protocol obtained ethical approval by the Ethics Committee of NOVA Medical School | Faculdade de Ciências Médicas (NMS|FCM), NOVA University of Lisbon (nº113/2021/CEFCM). An overview of the experimental protocol is represented in Figure 2.

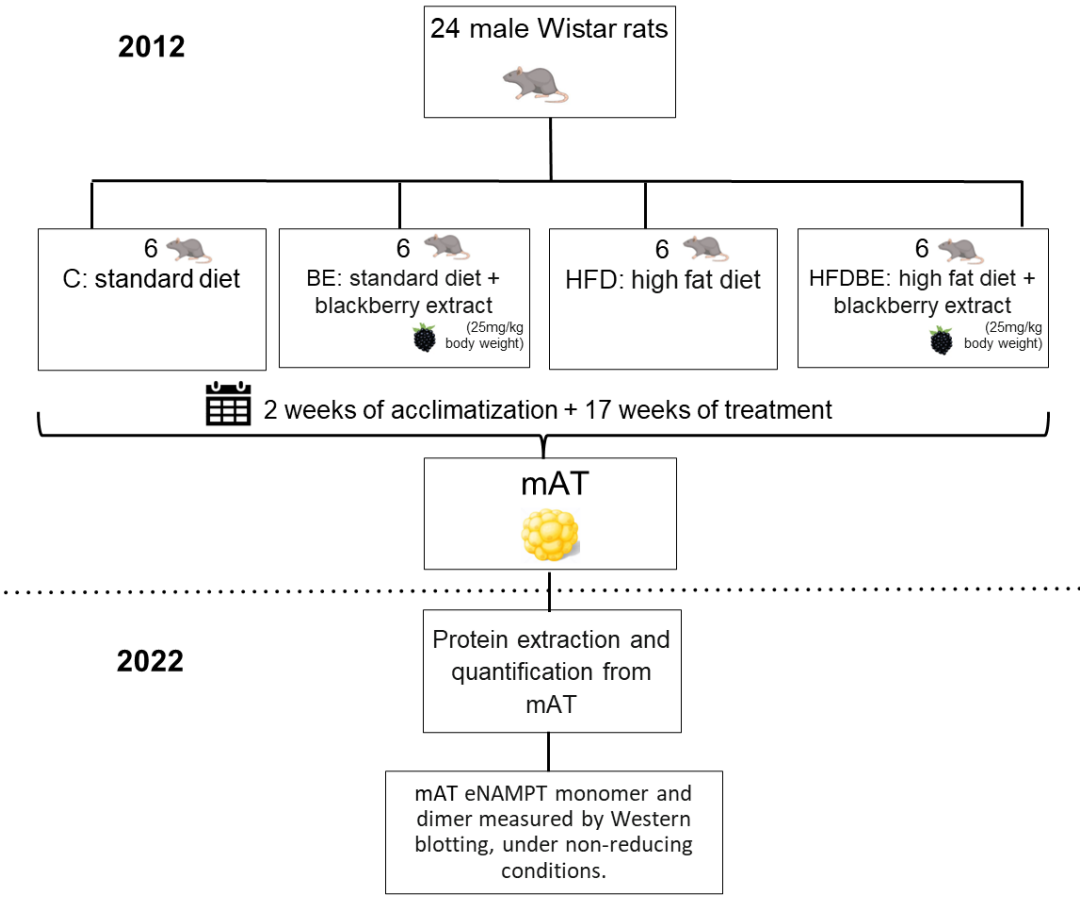


Figure 2 - Overview of experimental protocol and timeline. mAT - mesenteric Adipose Tissue.

3.2 Animals and Sample Collection

The mAT samples used in this study were previously collected during a research project in 2012 (62). Animal handling and housing protocols followed European Union guidelines (86/ 609/EEC) and the Portuguese Act (129/92) for the use of experimental animals. The study obtained ethical approval from the Ethical Committee of the Faculty of Medicine, University of Porto and the São João Hospital Center (46). Twenty-four male Wistar rats, acquired from Harlan Laboratories (Santiga, Spain), were divided into four groups (n = 6 rats per group), after two weeks of acclimatization, as follows: (C) standard diet; (BE) standard diet + blackberry extract; (HFD) high-fat diet; (HFDBE) high-fat diet + blackberry extract. Animals were fed *ad libitum* with "standard" (Teklad 2014, Harlan Laboratories, Santiga, Spain) or "high-fat" diets (D1245 Research Diets, New Brunswick, USA), according to their groups, for 17 weeks. Blackberry anthocyanin rich extract (25

mg/kg body weight/day) was dissolved daily in sterile water and embedded in food pellets that animals had daily access to. Animals were maintained at 23–25 °C with a 12/12 h light–dark cycle and housed two per cage. Food ingestion was measured twice a week. Fasting blood glucose, and glucose and insulin tolerance tests were also assessed then, and the detailed methodology is described in the paper from Meireles et al. 2016 (46). This data is presented in this thesis to contextualize further results obtained in this project and for interpretation purposes. After sacrifice, mAT was collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use. In the present study, protein extraction and quantification were performed from these mAT samples.

3.3 Protein Extraction

Approximately 300µg of each mAT sample were grounded in liquid nitrogen with a mortar and pestle and transferred to new Eppendorf tubes (1,5mL). Reconstructed Radio-Immunoprecipitation (RIPA) Lysis Buffer with proteases inhibitors (10µL PMSF solution, 10µL sodium orthovanadate solution, and 10-20µL protease inhibitor cocktail solution per mL of 1X RIPA Lysis buffer) (Cat#sc-24948A, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the tubes, following the supplier recommendations, and homogenized using an electric homogenizer. RIPA Buffer enables efficient tissue and cell lysis, and protein solubilization while avoiding protein degradation. The samples were refrigerated at -20°C, for at least 2h and then centrifuged at 10000 x g, for 15 min at 4°C. The tubes were gently removed from the centrifuge and placed on ice. The supernatant was aspirated from each sample (± 600 µL) and placed in fresh identified Eppendorf tubes kept on ice. The lipid upper phase and the pellet were discarded.

3.4 Protein Quantification

For protein quantification, an EZQ® Protein Quantitation Kit (R33200, Thermo Fisher Scientific, USA) was used. After preparing the microplate cassette with the assay paper, 1 well was left with nothing to ensure non-contamination of the assay paper (negative control 1), and 1µL of reconstructed RIPA Lysis Buffer was spotted onto another well (negative control 2). The samples were spotted onto assay paper in triplicate using 1µL of each protein extraction sample per well (protein extract was not diluted to avoid loss of protein). The paper was dried and fixed with methanol for 5 minutes with gentle agitation to remove contaminating substances. The EZQ® protein quantitation reagent was added into a staining tray and the protein-spotted assay paper was added into the stain solution and agitated gently on an orbital shaker for 30 minutes. After staining, the assay paper was washed twice for 1–2 minutes with a rinse buffer solution (10% methanol, 7% acetic acid) and after completely dried was replaced into the microplate. The fluorescence was measured in a SpectraMax i3x plate reader (Molecular Devices; Sunnyvale, CA) using excitation/emission settings of 450/618nm. The final protein concentration was determined using a standard curve of ovalbumin (Appendix 1), and the samples were frozen at -20°C for later use. The samples had a very low protein concentration, insufficient to provide enough protein to

perform the Western blots (WB). In order to concentrate the samples, they were lyophilized overnight and resuspended in a smaller volume of ultrapure water (Milli-Q® systems). Protein quantification was then repeated according to the method described above.

3.4 eNampt Measurements - Protein Gel Electrophoresis and Western Blotting

In order to separate proteins, a sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of 4-15% precast polyacrylamide gels (#4568034, Bio-Rad Laboratories Inc, USA) was used. SDS is an anionic detergent, used to denature tertiary and secondary protein structures, thus allowing a protein separation by size/molecular weight depending on their electrophoretic mobility in a polyacrylamide gel. After normalizing to 15µg of protein, adding 10µL of loading buffer (6X, 400/L saccharose, 2,5g/L bromophenol blue) and ensuring an equivalent volume of 40µL to load each lane, samples were heated at 100°C for 10 minutes and centrifuged at 4,000 rpm for 20 seconds. No reducing agent was added to the protein samples prior to SDS-PAGE to allow detection of both monomeric and dimeric eNAMPT in biological samples. Before loading the samples, 3µL of a prestained protein ladder (R26619, PageRuler™Plus Thermo Fisher Scientific, USA) was loaded to one of the wells of each gel to serve as a size reference to which samples could be compared later. 40µL of the respective samples were loaded to the remaining gel wells, and electrophoresis was run at 100V for 90 minutes with migration/running buffer (3g/L TRIZMA® base, 14g/L glycine, 10mL/L SDS 10%) in mini-PROTEAN® Tetra system gel tanks (Bio-Rad Laboratories Inc, USA). The evolution of electrophoresis was monitored by observing the migration of the bromophenol blue dye that was added to the loading buffer. Due to its low molecular weight and negative charge, it migrates along with proteins towards the positive anode.

After the proteins have been separated by gel electrophoresis, the WB technique requires the transfer of the proteins to a membrane, in this case a polyvinylidene difluoride (PVDF) membrane, for subsequent densitometric quantification. PVDF membranes are highly hydrophobic, so prior to their utilization they were pre-wetted with ethanol for 1 minute, water for 10 minutes, and at least 10 minutes with transfer buffer (1.5g/L TRIZMA® base, 7.5g/L glycine). The transfer devices, with the black part of the cassettes facing bottom, were prepared in the following way for each cassette: 1 scratch sponge, 2 Whatman® blotting papers, SDS-PAGE gel, PVDF membrane, 2 Whatman® blotting papers, 1 scratch sponge, and everything was embedded in transfer buffer. The transfer cassettes were then inserted into the Mini Trans-Blot Cell® buffer tank (Bio-Rad Laboratories Inc, USA), which was filled with transfer buffer, and blotting was performed at 50 V for 95 minutes, so the negatively charged proteins could transfer onto the membrane, by migrating towards the positively charged electrode of the blotting chamber. The efficiency of transfer was controlled by the presence of the protein ladder on the membrane at the end.

Following the transfer, membranes were washed twice with phosphate-buffered saline (PBS) containing 0,1% (vol/vol) Tween-20 (TPBS). Membranes were then blocked with 5% (wt/vol) dried

skimmed milk (Molico®) dissolved in TPBS (2,5 g per 50mL) for 60 minutes at room temperature under agitation, in order to prevent non-specific binding during the following antibody incubations. After quickly rinsing the blocked membranes three times with TPBS, a mouse primary antibody against NAMPT (Cat#sc-393444, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 dilution in TPBS containing 5% BSA) was incubated with the membranes overnight at 4°C on a rocking platform shaker. Membranes were again washed three times with TPBS, with gently agitation on an orbital shaker for 10 minutes each time, to eliminate unbound antibody residues and posteriorly incubated with secondary antibody anti-mouse conjugated to horse-radish peroxidase (HRP) (Cat#sc-542741, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:3000 dilution in TPBS containing 5% BSA) for 60 minutes at room temperature with gentle agitation. Following one quick wash with TPBS, membranes were again washed three times with TPBS, with gently agitation on an orbital shaker for 10 minutes each time.

The proteins were then visualized in a ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories Inc, USA) after the membranes were washed for 1 minute with a chemiluminescent WB detection reagent (RPN2235, Amersham™ ECL Select™, cytiva), which allows the HRP to produce a luminescent signal. The signal obtained is proportional to the antibody-bounded protein, which appears as bands, therefore is used as the measurement for protein quantity. Densitometry of WB bands was calculated using Fiji software version V1.52 E.

3.5 Statistical Analysis

The normality of the data was assessed by a Shapiro-Wilk test. Comparison between C vs HFD, C vs BE and HFD vs HFDBE groups was performed using unpaired t-tests. One way-ANOVA was undertaken to analyse the variance across the groups. Tukey's post hoc test was then implemented to verify potential differences between the treatment groups. Data are expressed as mean ± standard error of the mean (SEM). Results were considered statistically significant for $p \leq 0.05$. All statistical analysis were performed in GraphPad Prism 8 software (GraphPad Software, San Diego, USA).

4. RESULTS

4.1 Data previously collected, analysed and described in Meireles et al. 2016

The animal experiments originally took place in 2012. All animals started the diet treatment at similar body weights (Table 1). The high fat diets - HFD and HFDBE - resulted in overweight rats, with increased body fat percentage. The effect of blackberry anthocyanin extract supplementation on body weight gain was not significant (62).

	C	BE	HFD	HFDBE
Initial body weight (g)	237,8±7,7	246,5±8.6	237,2±7.2	244,0±11,1
Final body weight (g)	429,8±27,8[a]	432,7±28,4[a]	550,7±67,6[b]	581,0±63,1[b]

Table 1 –Body weight of rats from different groups before (initial body weight) and after the 17 weeks of diet and blackberry anthocyanin extract supplementation (final body weight). Values are expressed as mean ± Standard Deviation (SD). Different superscript letters represent statistically significant differences ($p<0,05$): C – standard diet; BE – standard diet + blackberry extract; HFD – high-fat diet; HFDBE – high-fat diet + blackberry extract. Reproduced from Meireles et al., 2016 to contextualize further results obtained in the project here presented.

Fasting blood glucose was assessed in these animals, showing significant differences between groups ($p<0,05$). The HFDBE had higher fasting glucose levels compared to C at the 15th week of treatment. (*$p<0,05$) (figure 3).

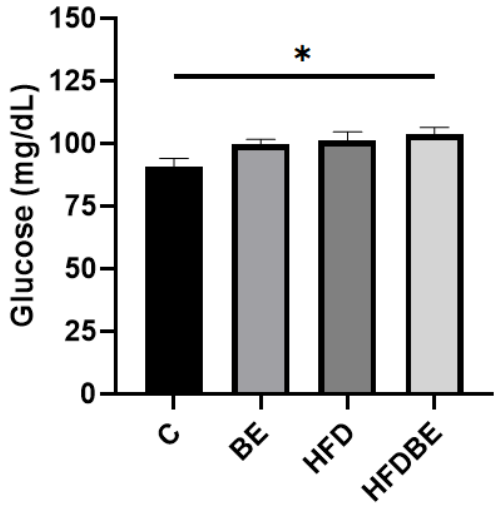


Figure 3 – Fasting blood glucose levels of rats from the four groups (n = 6). Data are expressed as means ± SEM. Statistical significance was tested by one-way ANOVA, followed by a Tuckey's post hoc analysis for comparison between groups ($p<0,05$). The HFDBE had higher fasting glucose levels compared to C (*$p<0,05$). Reproduced from Meireles et al., 2016 to contextualize further results obtained in the project here presented.

Glucose tolerance was also tested in these animals at weeks 8 and 15, during the treatment (Figure 4). After the oral gavage of a solution with 2 g per kg weight of glucose, the glycemic response was evaluated at 15, 30, 60, 90, and 120 minutes (46). The increase of total area under the curve (AUC) at the 15th week of treatment shows an impairment of the glucose sensibility in animal fed a HFD (Figure 4a). Animals from BE group showed a tendency to a lower AUC, significant at the 8th week of treatment ($p<0,05$, Figure 4b). However, after 15 weeks, the glucose tolerance response was significantly affected by diet ($p<0,05$, figure 4c), but not by blackberry extract supplementation (46).

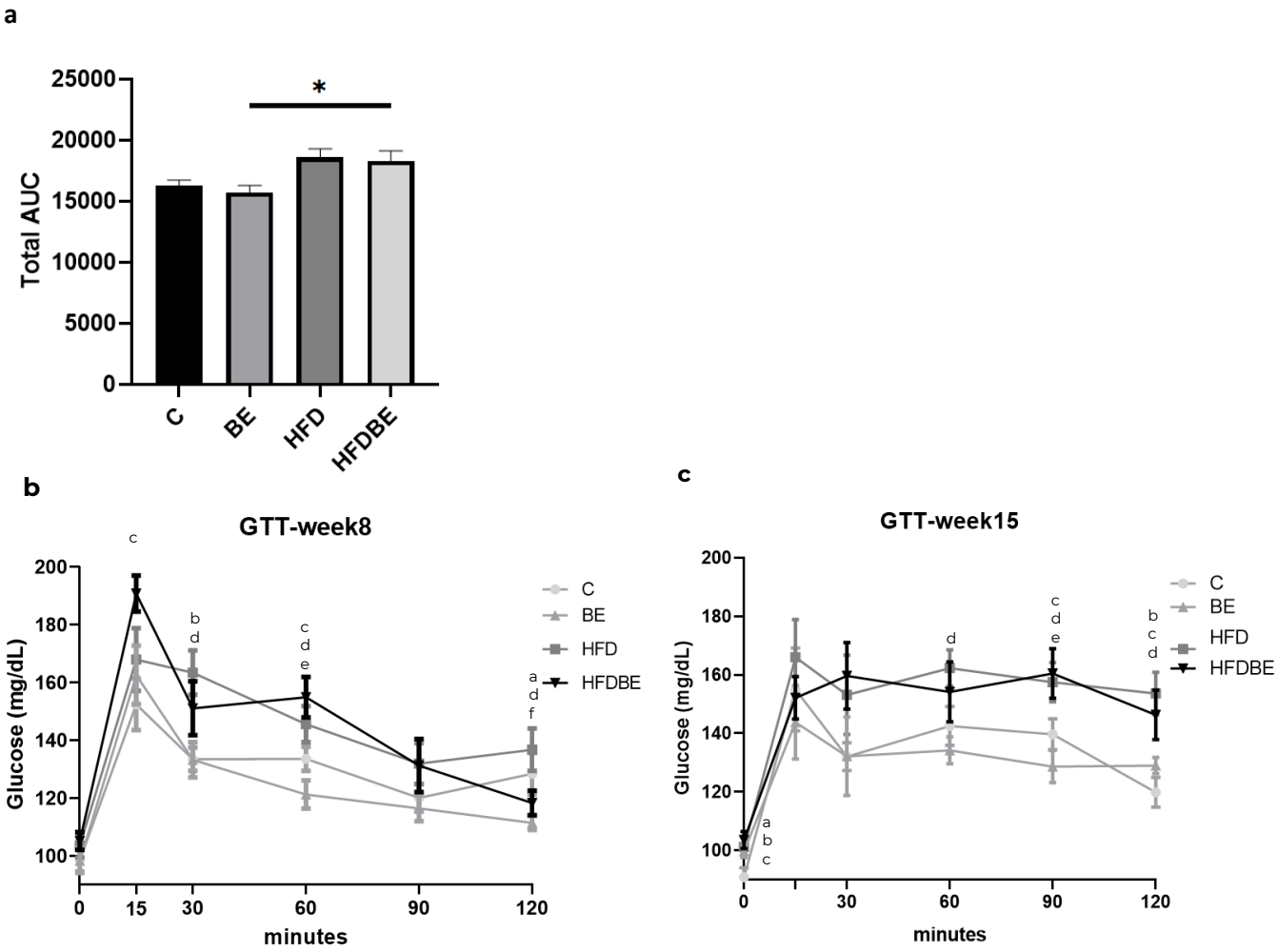


Figure 4 - Effect of blackberry anthocyanin extract supplementation on glycemic response to oral glucose tolerance test in C and HFD rats. a) Area under the curve of total glycemic response over 120 minutes following the oral glucose tolerance test (GTT) at the 15th week of treatment (*n* = 6). b) glycemic response at each time point during the 120 minutes at the 8th week of treatment. c) glycemic response at each time point during the 120 minutes at the 15th week of treatment. Letters represent the following differences (a) C vs. BE; (b) C vs. HFD; (c) C vs. HFDBE; (d) BE vs. HFD; (e) BE vs. HFDBE; (f) HF vs. HFDBE. Statistical significance was tested by two-way ANOVA, considering time and diet as factors, followed by a Fisher's LSD test. Superscript letters represent statistical differences between means ($p<0,05$). Data reproduced from Meireles et al., 2016 to contextualize further results obtained in the project here presented.

Glycemic levels during insulin tolerance tests were also evaluated. A solution of insulin (0,5 U per kg weigh) was administered by intraperitoneal injection (46). After the 15th week of treatment HFBE rats had a better sensitivity to insulin than HFD rats (Figure 5)(46).

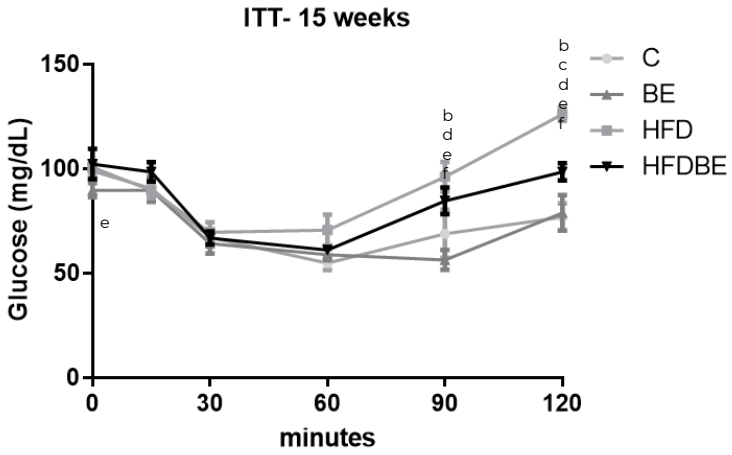


Figure 5 - Effect of blackberry anthocyanin extract supplementation on glycemic response to insulin tolerance test (ITT) in C and HFD rats at 15th week of treatment. Letters represent the following differences (a) C vs. BE; (b) C vs. HFD; (c) C vs. HFDBE; (d) BE vs. HFD; (e) BE vs. HFDBE; (f) HF vs. HFDBE. Statistical significance was tested by two-way ANOVA, considering time and diet as factors, followed by a Fisher's LSD test. Superscript letters represent statistical differences between means ($p<0,05$). Data reproduced from Meireles et al., 2016 to contextualize further results obtained in the project here presented.

4.2 Effect of a HFD in mAT eNAMPT monomer expression levels

mAT from HFD animals displayed higher levels of eNAMPT monomer, compared to the C animals (138,6% ± 11,4% HFBE vs 100% C, p=0,01; Figure 6).

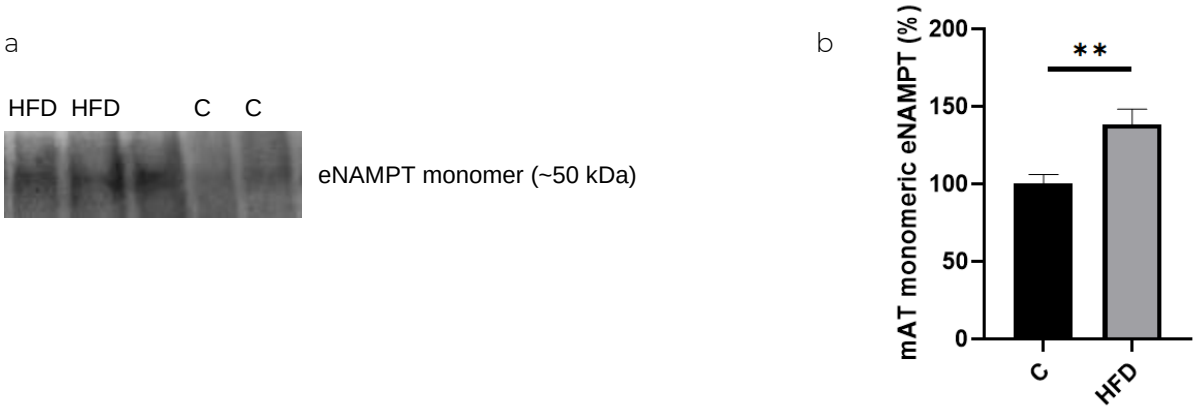


Figure 6 - eNAMPT monomer levels determined by Western blot. a) representative image of WB of mAT eNAMPT monomer of C and HFD treated animals; b) Graphic representing expression of mAT eNAMPT monomer in HFD group when compared to C (HFD quantified as % of controls [levels in C group]) (*n* = 5). Data was collected and analysed from three separate blots. Data are expressed as means ± SEM. Statistical significance was tested by t-test and consider significant when $p<0,05$ (**p=0,01).

4.3 Effect of anthocyanin treatment in mAT eNAMPT monomer expression levels

The anthocyanin's treatment influence on mAT eNAMPT monomer expression was also assessed with significant results in the ANOVA test (p=0.05). After conducting the post-hoc analysis, we found that eNAMPT-monomer showed a tendency to have higher expression in rats fed HFD compared to HFDBE (60,4% [-1,2, 122,1], p= 0.06) (Figure 7b).

In order to better understand the differences in the effect of the anthocyanin treatment in and out of the obesogenic context, statistical significance was tested by unpaired t-tests. mAT eNAMPT monomer expression was significantly decreased in the HFDBE group compared to HFD alone (-54,1 ± 15,3 % [-89.4, -18.8], p<0,01). No differences were seen between the other groups. (Figure 7c,d).

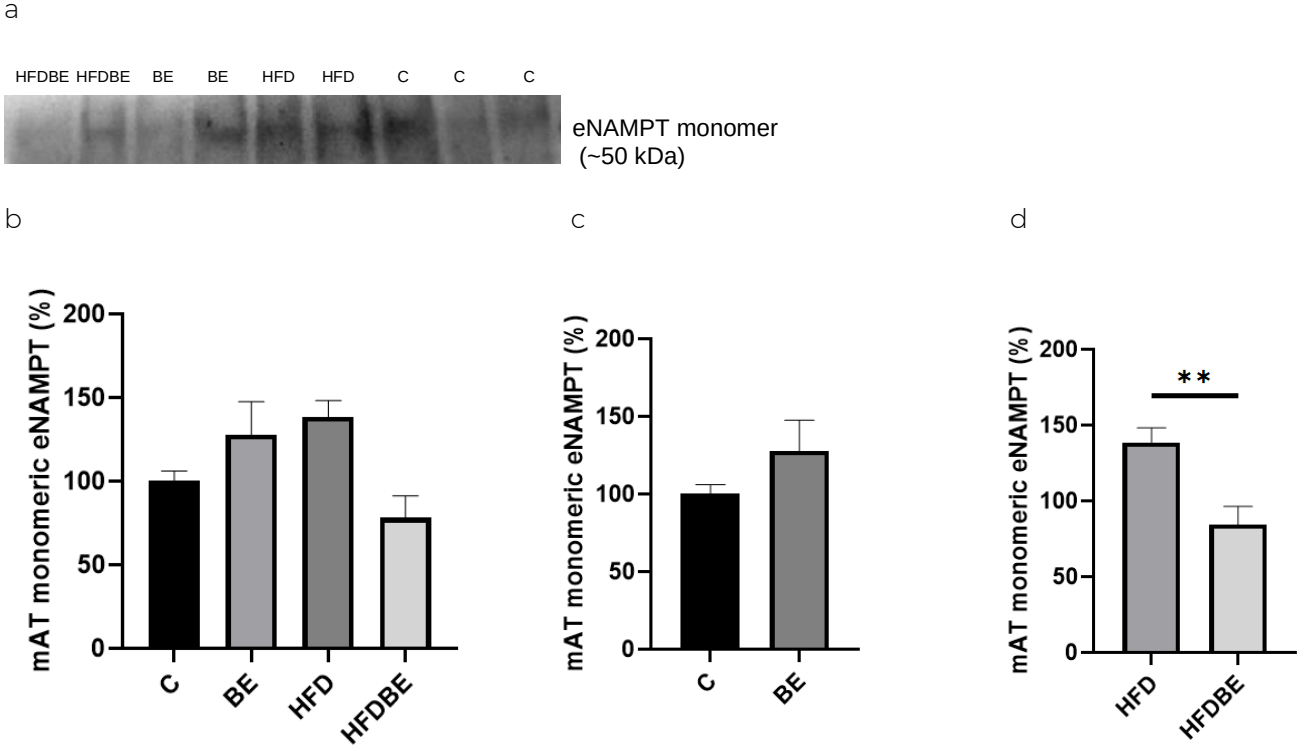


Figure 7 - Effect of blackberry anthocyanin extract supplementation on mAT's eNAMPT monomer expression. a) Western blot of mAT eNAMPT monomer of C, BE, HFD and HFDBE groups; b) Graphic representing mAT eNAMPT monomer expression in C (*n* = 5), BE (*n* = 6), HFD (*n* = 5) and HFDBE (*n* = 4) groups (expressed as % of controls [levels in C rats]). Statistical significance was tested by one-way ANOVA, followed by a Tuckey's post hoc analysis for comparison between groups (p=0,05). c-d) Graphic representing mAT eNAMPT monomer expression in C vs BE and HFD vs HFDBE (expressed as % of controls [levels in C rats]). Statistical significance was tested by t-test and consider significant when p<0,05 (**p=0,01). Data was collected and analysed from three separate blots. Data are expressed as means ± SEM.

4.4 Differences in eNAMPT's expression, in its dimeric versus monomeric forms

We were unable to analyse the expression of mAT eNAMPT dimer as we proposed, since the technique was not sufficiently sensitive to obtain high quality images, needed for quantification.

5. DISCUSSION

With this study, we provide lines of evidence that suggest and support a relation between anthocyanin consumption and the eNAMPT monomer expression, which may be a potential mechanism supporting the halt of the progression of obesity-associated comorbidities, particularly concerning glucose metabolism and T2D.

This research aimed to investigate the differences in mAT eNAMPT's expression from an animal model in or out of a context of a diet-induced obesity. The original project that led to the study here presented demonstrated that an HFD resulted in an increased body weight (62) and other metabolic complications assessed by our working group, such as impairment in glucose tolerance and insulinemic response, increase in adipokine production – adiponectin and leptin – and increase in scAT adipocyte size (46). Using mAT samples from such study, we were able to observe that the HFD promoted a higher expression of mAT eNAMPT monomer compared to controls (p=0,01). Several reports have shown significant associations between obesity, especially visceral fat, and overexpression of NAMPT (33,70–72). A cross-sectional study in children with obesity has shown that eNAMPT concentration was 2-fold higher in those than in healthy controls, but this was not directly correlated with BMI (71). Sandeep et al. also demonstrated that eNAMPT had a significant association with obesity, in humans (33). Particularly visceral fat, but not subcutaneous fat, was significantly associated with eNAMPT levels even after adjusting for age, sex, T2D, and BMI (33). In obesity, as AT undergoes structural and metabolic changes, there is a chronic inflammatory environment derived from the abnormal production and release of pro-inflammatory cytokines by this tissue (4). The obese adipose tissue already has a representative amount of macrophages and being themselves pro-inflammatory cytokine releasers, plus the fact that adipocytes have the ability to recruit monocytes by chemoattractants, leads to increased inflammatory activity in this area. This over-activation of inflammation in AT appears to underlie the metabolic dysfunction associated with obesity (9). Adipocyte dysfunction, with ultimate dysregulation of adipokines, particularly eNAMPT, contributes to obesity-associated metabolic disorders. However, the mechanisms behind the pathogenesis of these obesity-associated metabolic complications are complex and still unclear. In fact, evidence is still controversial and the mechanisms by which eNAMPT exerts proinflammatory properties or promotes and protects a physiological metabolism is still under investigation. Evidence has shown that the catalytic activity of eNAMPT depends on its dimerization (15), however more recently, eNAMPT has also been shown to exist in a monomeric form, which has been related to the pro-inflammatory activity and the deleterious effects involved in beta cell dysfunction in T2D (40,73). Yoshino et al. showed that both NAMPT protein and NAD levels were reduced in HFD-induced obese mice's metabolic tissues and organs – liver and WAT - , contributing to the pathogenesis of T2D, which developed in these animals (45). However, the authors did not specify whether it was the dimeric or monomeric form. In the same study, NMN administration strongly ameliorated impaired glucose tolerance by restoring normal NAD levels and enhancing either IS or insulin secretion

(45). Consequently, one may hypothesize that it was the dimeric form of eNAMPT, capable of biosynthetic functions, that was diminished. Another group found changes in iNAMPT, eNAMPT and inflammatory levels in mice fed a fructose-rich diet (FRD). FRD increased iNAMPT abundance in AT while suppressing circulating eNAMPT concentrations and insulin secretion (74). eNAMPT not being secreted leads to a reduction of circulating NMN, which has been shown to be essential for beta cell function. In vivo administration of NMN abolished the suppressive effects of FRD on GSIS (Figure 8) (74). Once again, the authors did not specify whether these lower levels of serum eNAMPT were related to the dimeric form, nor did they assess the levels of the monomeric form.

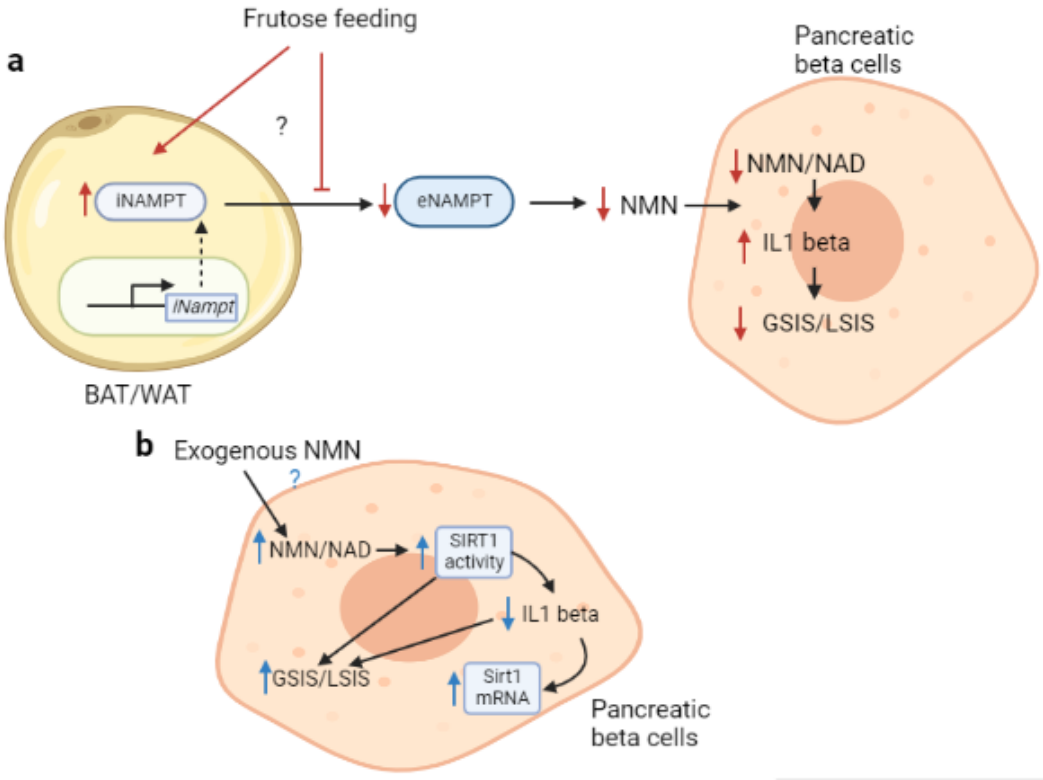


Figure 8 – Schematic proposal of eNAMPT's release suppression and NMN deficiency effects on beta cell function. a. FRD fed mice shows a suppression of eNAMPT's release from WAT, resulting in reduced plasma eNAMPT levels and consequent reduction of NMN. This reduction will interfere with beta-cells function, resulting in a suppressed GSIS. b. Exogenous administration of NMN leads to an increase in SIRT1's activity that restores GSIS. Adapted from Caton et al., 2011.

More recently, studies have been conducted to access and understand these structural and functional changes of eNAMPT protein. A study using two mouse models – diabetics on a HFD and lean non-diabetics on a control diet – showed that serum and WAT monomeric eNAMPT levels were significantly higher in the diabetic mouse model ($p<0,05$ and $p<0,01$, respectively) (73). Serum dimeric eNAMPT levels did not significantly change between the two groups, although in WAT it significantly decreased in HFD-fed mice ($p<0,05$). Furthermore, these diabetic mice

improved all profiles associated with the condition, after receiving an antibody against the monomeric eNAMPT, such as decreased blood glucose, improved glucose tolerance and IR, improved pancreatic islet function, and reduced inflammation (73). On the other hand, upon administration of eNAMPT-monomer to the non-diabetic mouse model, they developed a diabetic phenotype, characterized by an increase in blood glucose, impaired glucose tolerance and insulin secretion and by the presence of a generalized inflammation, without changes in NAD levels (73). These results suggest that these pro-inflammatory and diabetic effects are independent of eNAMPT's NAD biosynthetic activity, providing evidence that this monomeric form not only does not present enzymatic capacity, but is also responsible for the previously mentioned adverse effects.

In agreement with the aforementioned study, other analysis conducted in individuals with obesity, diabetic and non-diabetic, for detection of eNAMPT's dimeric and monomeric levels, showed that serum eNAMPT concentrations increased with progression of T2D. eNAMPT monomer was found to be significantly higher in T2D (40). These increasing concentrations did not correlate with serum NMN levels. This study also showed that eNAMPT concentration was a critical factor in these changes in structure and function. At low and physiological concentrations (1 ng/ml) eNAMPT remained in its dimeric form, optimizing beta cell function through its NAD-biosynthetic capacity. However, at supraphysiological levels (5 ng/ml), characteristic of obesity and T2D, eNAMPT adopted a monomeric structure and deterioration of beta cell function was observed, resulting from pro-inflammatory mechanisms independent of NAD (40).

Although is not yet clear how and why these structural differences occur during the rising concentrations, studies present different hypotheses, being one of them the possible necessity of a ligand-induced dimerization, that begins to be deficient at higher eNAMPT concentrations, leading to the breakup of the dimer into constitutive monomers (73). The other possibility described involves an obesity-mediated dysfunctional AT that is characterized by poorly differentiated adipocytes and immune cells, incapable of secreting eNAMPT dimer, as this form is said to be secreted by fully differentiated adipocytes (40). Either way, finding approaches that can stabilize eNAMPT in dimer form, by controlling its rising serum concentration, or that can block the actions of the monomer form, by neutralizing eNAMPT monomer appears to be a promising future for disease prevention and potentially contributing to the therapeutic strategies for metabolic disorders such as obesity and T2D.

The main purpose of this study was also to analyse the impact of an anthocyanin treatment in eNAMPT's expression on mAT of these animal model of increased metabolic risk. Diet is a key factor in terms of managing the risk of obesity and obesity-related disfunctions. Previous studies have shown that a dietary intervention with anthocyanins is effective in reducing the risk of obesity and preventing or treating obesity-related co-morbidities (66,67). The results in subjects

with obesity suggested that daily intake of dietary anthocyanins might aid in maintaining or reducing the body weight and attenuating some risk factors of metabolic syndrome (75,76).

The anthocyanin extract from blackberry given to the animals in this study was able to attenuate the raise of mAT eNAMPT monomer in the context of an HFD. mAT eNAMPT monomer expression was significantly decreased in group HFDBE compared to HFD (-54,1 ± 15,3 % [-89.4, -18.8], p<0,01). In support of these results, a previous randomized placebo-controlled trial also aimed to examine the effects of purified anthocyanins supplementation on eNAMPT and another adipokine in Chinese adults with prediabetes and newly diagnosed, untreated T2D (69). Supplementation with purified anthocyanins for 12 weeks decreased serum eNAMPT in patients with prediabetes or newly diagnosed diabetes and showed improvements in glucolipid metabolic parameters (69). A significant difference in serum eNAMPT (-3,5 ng/mL [-6,69, -0,31], p=0,03) was observed between the anthocyanins and placebo groups, however it is not mentioned if it is the monomeric or dimeric form that has been evaluated. Serum eNAMPT decreased by 9,7% (-3,57 ng/mL [-5,98, -1,15], p=0.004) after anthocyanins treatment compared to the 0,2% decrease (-0,07 ng/mL [-2,07, 1,94], p=0,947) after placebo treatment (69). The authors had already demonstrated that anthocyanins inhibited the secretion of eNAMPT in a previous animal study (8), and an important conclusion from that experiment was that Cy-3-G, as the most abundant monomer of anthocyanins, could regulate the secretion of monomeric eNAMPT from brown adipocytes. After being incubated with palmitate, the release of monomeric eNAMPT from those cells was increased and could be inhibited by Cy-3-G (8). The main anthocyanin in blackberry is Cy-3-G (about 85%). As such, the anthocyanin extract from blackberry used for treating the animals on the project that led to our study is rich in Cy-3-G.

As assessed by our working group in the original project from Meireles et al 2016, diet was a significant factor that influenced the glucose sensibility of the animals (46). The HFD showed a tendency to promote a higher fasting glucose in animals, an observation that was not attenuated by the anthocyanin treatment. The HFDBE had higher fasting glucose levels compared to C (p<0,05). As these animals gained weight along the weeks of treatment, especially the HFD groups, the risk of greater insulin resistance and glucose intolerance was higher, as these conditions are highly associated with obesity. As expected, animals from the HFD displayed a lower response to insulin as well as an impaired glucose sensibility (46). As discussed earlier, reductions in plasma biosynthesis of NMN, result in severe impairments in IS and GSIS (35). With this study we demonstrated that an obesogenic condition, imposed by a HFD, led to an increase in the monomeric eNAMPT in these animal's mAT. This setting may have led to a greater secretion of the less biosynthetic monomeric eNAMPT into the circulation and consequently, to a decrease in serum NMN, being this the possible cause for the derangements in the target tissues most susceptible to these alterations, such as beta cells (Figure 9). However, the dimeric form of eNAMPT should have been analysed for a better comprehensive analysis of the results, including the ratio monomer/dimer instead of the absolute quantification of the monomeric eNAMPT

alone. If the ratio had increased, as would be expected at the outset, this would lead to a decrease in the production of NMN, as seen in other studies (40,73,74).

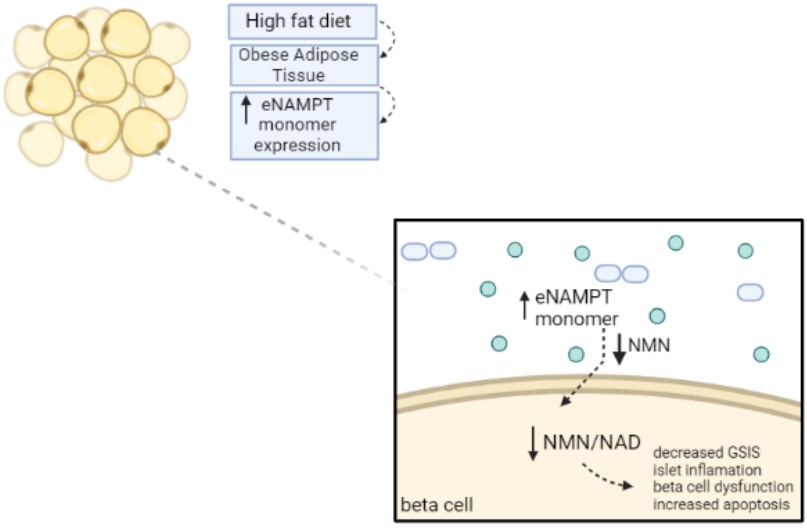


Figure 9 - Schematic proposal of eNAMPT monomer's higher expression in obese adipose tissue and the consequent NMN deficiency effects on beta cell function. An obesogenic condition, imposed by a HFD, led to an increase in the monomeric eNAMPT in these animal's mAT. This setting may have led to a greater secretion of the less biosynthetic monomeric eNAMPT into the circulation and consequently, to a decrease in serum NMN, being this the possible cause for the derangements in the target tissues most susceptible to these alterations, such as beta cells.

Nonetheless, to a certain extent supplementation with blackberry anthocyanin extract was able to attenuate the impairment of the glycemic response caused by the HFD, as seen in the AUC of the HFDBE group, after 8 weeks. A possible hypothesis is that as the anthocyanin's treatment was able to concurrently attenuate the increase of the mAT monomeric form of eNAMPT, as demonstrated in this study, the dimeric form may have been secreted in higher or normal levels from the AT in this group of animals, maintaining serum NMN levels adequate and as so protecting the beta cell from the deleterious effects previously discussed, particularly attenuating the impairment of the glucose sensibility, and optimizing the insulin response. After the 15th week of treatment HFBE group also had a better response to insulin than HFD rats ($p<0,05$).

Together these data demonstrate that mAT levels of eNAMPT monomer were selectively elevated in HFD-fed mice, but the anthocyanin's treatment was able to significantly minimize/abrogate this abnormal increase.

However, this relation requires further investigation. It is necessary to carry out studies with a larger *n* and preferably a human study design to prove the hypothesis raised and deepen the technique that allows the quantification of the dimeric form, that can provide valuable

information. Being able to analyse both forms of eNAMPT would bring a more in-depth investigation of the molecular mechanisms resulting in the repercussions of the alterations in this adipokine. Further investigation will also be required to identify detailed mechanisms for the efficacy of the anthocyanins. Additional interventional studies, preferably long-term trials are needed to clarify and confirm our findings and test the potential of blackberry supplementation as a therapeutic intervention in humans.

6. CONCLUSIONS

Despite NAMPT's essential role in metabolism through its catalytic function in the rate-limiting step in NAD biosynthesis, the protein has become the center of attention due to the subsequent association of its monomeric-non-biosynthetic form with metabolic and inflammatory diseases. This study demonstrates that an anthocyanin treatment abrogates the elevation of monomeric eNAMPT protein in mAT from animals inflicted with a HFD - that negatively impacted their metabolism. Here we hypothesize that the anthocyanin treatment, by reducing the mAT monomeric eNAMPT levels, was able to attenuate some of the resulted impairments, such as glucose intolerance, and optimize the response to insulin.

Ultimately, our results suggest that long-term anthocyanin treatment/supplementation might be effective for sustaining lower levels of monomeric eNAMPT in the context of diet-induced obesity, potentially preventing or delaying the consequent metabolic impairments as the alarming epidemic of T2D. Nevertheless, these were studies performed in a pre-clinical model of obesity and clinical trials are needed to clarify such relationships. However, the consumption of anthocyanins, through a diet rich in fruits and vegetables, particularly through red fruits, can be an interesting dietary approach to implement and/or emphasize in clinical practice, given the existing beneficial evidence reinforced by this work.

7. REFERENCES

1. Sun K, Kusminski CM, Scherer PE, Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity Find the latest version : Review series Adipose tissue remodeling and obesity. 2011;121(6):2094–101.
2. Cao H. Adipocytokines in obesity and metabolic disease. Journal of Endocrinology [Internet]. 2014 Feb;220(2):T47–59. Available from: https://joe.bioscientifica.com/view/journals/joe/220/2/T47.xml
3. Moschen AR, Kaser A, Enrich B, Mosheimer B, Theurl M, Niederegger H, et al. Visfatin, an Adipocytokine with Proinflammatory and Immunomodulating Properties. The Journal of Immunology. 2007;178(3):1748–58.
4. Mohammadi M, Mianabadi F, Mehrad-Majd H. Circulating visfatin levels and cancers risk: A systematic review and meta-analysis. J Cell Physiol. 2019;234(4):5011–22.
5. Flehmig G, Scholz M, Klöting N, Fasshauer M, Tönjes A, Stumvoll M, et al. Identification of adipokine clusters related to parameters of fat mass, insulin sensitivity and inflammation. PLoS One. 2014;9(6).
6. Lago F, Gómez R, Gómez-Reino JJ, Dieguez C, Gualillo O. Adipokines as novel modulators of lipid metabolism. Trends Biochem Sci. 2009;34(10):500–10.
7. Cannon B, Nedergaard J. Brown Adipose Tissue: Function and Physiological Significance. 2004; Available from: www.prv.org
8. Pei L, Wan T, Wang S, Ye M, Qiu Y, Jiang R, et al. Cyanidin-3-O-β-glucoside regulates the activation and the secretion of adipokines from brown adipose tissue and alleviates diet induced fatty liver. Biomedicine and Pharmacotherapy [Internet]. 2018;105(74):625–32. Available from: https://doi.org/10.1016/j.biopha.2018.06.018
9. Tilg H, Moschen AR. Role of adiponectin and PBEF/visfatin as regulators of inflammation: Involvement in obesity-associated diseases. Clin Sci. 2008;114(3–4):275–88.
10. Longo M, Zatterale F, Naderi J, Parrillo L, Formisano P, Raciti GA, et al. Adipose tissue dysfunction as determinant of obesity-associated metabolic complications. Int J Mol Sci. 2019 May 1;20(9).
11. Kawai T, Autieri M v., Scalia R. Adipose tissue inflammation and metabolic dysfunction in obesity. Am J Physiol Cell Physiol. 2021 Mar 1;320(3):C375–91.
12. Zwick RK, Guerrero-Juarez CF, Horsley V, Plikus M v. Anatomical, Physiological, and Functional Diversity of Adipose Tissue. Vol. 27, Cell Metabolism. Cell Press; 2018. p. 68–83.
13. Imai S ichiro. Nicotinamide Phosphoribosyltransferase (Nampt): A Link Between NAD Biology, Metabolism, and Diseases. Curr Pharm Des [Internet]. 2009 Jan 1;15(1):20–8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf
14. Imai S, Yoshino J. The importance of NAMPT/NAD/SIRT1 in the systemic regulation of metabolism and ageing. Diabetes Obes Metab [Internet]. 2013 Sep;15(s3):26–33. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf
15. Revollo JR, Körner A, Mills KF, Satoh A, Wang T, Garten A, et al. Nampt/PBEF/Visfatin Regulates Insulin Secretion in β Cells as a Systemic NAD Biosynthetic Enzyme. Cell Metab [Internet]. 2007 Nov;6(5):363–75. Available from: https://linkinghub.elsevier.com/retrieve/pii/S155041310700263X
16. Belenky P, Bogan KL, Brenner C. NAD+ metabolism in health and disease. Trends Biochem Sci. 2007;32(1):12–9.
17. Yang L, Qiu Y, Ling W, Liu Z, Yang L, Wang C, et al. Anthocyanins regulate serum adipsin and visfatin in patients with prediabetes or newly diagnosed diabetes: a randomized controlled trial. Eur J Nutr [Internet]. 2020; Available from: https://doi.org/10.1007/s00394-020-02379-x
18. Grolla AA, Travelli C, Genazzani AA, Sethi JK. Extracellular nicotinamide phosphoribosyltransferase, a new cancer metabokine. Br J Pharmacol [Internet]. 2016 Jul 2;173(14):2182–94. Available from: https://joe.bioscientifica.com/view/journals/joe/220/2/T47.xml
19. Li H, Bai E, Zhang Y, Jia Z, He S, Fu J. Role of Nampt and Visceral Adiposity in Esophagogastric Junction Adenocarcinoma. J Immunol Res. 2017;2017.

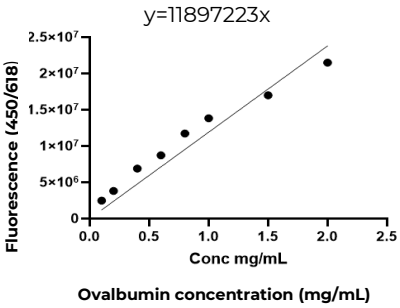
20. Brown JEP, Onyango DJ, Ramanjaneya M, Conner AC, Patel ST, Dunmore SJ, et al. Visfatin regulates insulin secretion, insulin receptor signalling and mRNA expression of diabetes-related genes in mouse pancreatic β-cells. J Mol Endocrinol. 2010;44(3):171–8.
21. R. Moschen A, R. Gerner R, Tilg H. Pre-B Cell Colony Enhancing Factor/NAMPT/Visfatin in Inflammation and Obesity- Related Disorders. Curr Pharm Des. 2010;16(17):1913–20.
22. Camp SM, Ceco E, Evenoski CL, Danilov SM, Zhou T, Chiang ET, et al. Unique Toll-Like Receptor 4 Activation by NAMPT/PBEF Induces NFκ B Signaling and Inflammatory Lung Injury. Sci Rep [Internet]. 2015;5(July):1–14. Available from: http://dx.doi.org/10.1038/srep13135
23. Oita RC, Camp SM, Ma W, Ceco E, Harbeck M, Singleton P, et al. Novel mechanism for nicotinamide phosphoribosyltransferase inhibition of TNF-α-mediated apoptosis in human lung endothelial cells. Vol. 59, American Journal of Respiratory Cell and Molecular Biology. 2018. p. 36–44.
24. Li Y, Zhang Y, Dorweiler B, Cui D, Wang T, Woo CW, et al. Extracellular nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism. Journal of Biological Chemistry. 2008;283(50):34833–43.
25. Wilson PWF, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. Circulation. 2005 Nov;112(20):3066–72.
26. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. Journal of Clinical Investigation. 2003 Dec 15;112(12):1796–808.
27. Porta C, Subhra Kumar B, Larghi P, Rubino L, Mancino A, Sica A. Chapter 5 Tumor Promotion by Tumor-Associated Macrophages.
28. Chang YH, Chang DM, Lin KC, Shin SJ, Lee YJ. Visfatin in overweight/obesity, type 2 diabetes mellitus, insulin resistance, metabolic syndrome and cardiovascular diseases: a meta-analysis and systemic review. Diabetes Metab Res Rev [Internet]. 2011 Sep;27(6):515–27. Available from: http://libweb.anglia.ac.uk/
29. Mohammadi M, Moradi A, Farhadi J, Akbari A, Pourmandi S, Mehrad-Majd H. Prognostic value of visfatin in various human malignancies: A systematic review and meta-analysis. Cytokine [Internet]. 2020;127(November 2019):154964. Available from: https://doi.org/10.1016/j.cyto.2019.154964
30. Luk T, Malam Z, Marshall JC. Pre-B cell colony-enhancing factor (PBEF)/visfatin: a novel mediator of innate immunity. J Leukoc Biol. 2008;83(4):804–16.
31. Lee YH, Bae SC. Circulating adiponectin and visfatin levels in rheumatoid arthritis and their correlation with disease activity: A meta-analysis. Int J Rheum Dis. 2018;21(3):664–72.
32. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, et al. Visfatin : A Protein Secreted by Visceral Fat That Mimics the Effects of Insulin Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/3840062 REFERENCES Linked references are available on JSTOR for th. Science (1979). 2005;307(5708):426–30.
33. Sandeep S, Velmurugan K, Deepa R, Mohan V. Serum visfatin in relation to visceral fat, obesity, and type 2 diabetes mellitus in Asian Indians. Metabolism. 2007;56(4):565–70.
34. Barth S, Klein P, Horbach T, Dötsch J, Rauh M, Rascher W, et al. Expression of neuropeptide Y, omentin and visfatin in visceral and subcutaneous adipose tissues in humans: Relation to endocrine and clinical parameters. Obes Facts. 2010;3(4):245–51.
35. Yoon MJ, Yoshida M, Johnson S, Takikawa A, Usui I, Tobe K, et al. SIRT1-Mediated eNAMPT Secretion from Adipose Tissue Regulates Hypothalamic NAD+ and Function in Mice. Cell Metab [Internet]. 2015 May;21(5):706–17. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1550413115001576
36. Esteghamati A, Alamdari A, Zandieh A, Elahi S, Khalilzadeh O, Nakhjavani M, et al. Serum visfatin is associated with type 2 diabetes mellitus independent of insulin resistance and obesity. Diabetes Res Clin Pract [Internet]. 2011;91(2):154–8. Available from: http://dx.doi.org/10.1016/j.diabres.2010.11.003
37. Chen MP, Chung FM, Chang DM, Tsai JCR, Huang HF, Shin SJ, et al. Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. Journal of Clinical Endocrinology and Metabolism. 2006;91(1):295–9.

38. Retnakaran R, Youn BS, Liu Y, Hanley AJG, Lee NS, Park JW, et al. Correlation of circulating full-length visfatin (PBEF/NAMPT) with metabolic parameters in subjects with and without diabetes: A cross-sectional study. Clin Endocrinol (Oxf). 2008;69(6):885–93.
39. López-Bermejo A, Chico-Julià B, Fernàndez-Balsells M, Recasens M, Esteve E, Casamitjana R, et al. Serum visfatin increases with progressive β-cell deterioration. Diabetes. 2006;55(10):2871–5.
40. Sayers SR, Beavil RL, Fine NHF, Huang GC, Choudhary P, Pacholarz KJ, et al. Structure-functional changes in eNAMPT at high concentrations mediate mouse and human beta cell dysfunction in type 2 diabetes. Diabetologia. 2020;63(2):313–23.
41. Bloomgarden ZT. Adiposity and diabetes. Diabetes Care. 2002;25(12):2342–9.
42. Mursu J, Virtanen JK, Tuomainen TP, Nurmi T, Voutilainen S. Intake of fruit, berries, and vegetables and risk of type 2 diabetes in Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. Am J Clin Nutr [Internet]. 2014 Feb 1;99(2):328–33. Available from: https://academic.oup.com/ajcn/article/99/2/328/4571480
43. Wu LLY, Dunning KR, Yang X, Russell DL, Lane M, Norman RJ, et al. High-fat diet causes lipotoxicity responses in cumulus - Oocyte complexes and decreased fertilization rates. Endocrinology. 2010;151(11):5438–45.
44. Scha. Lipotoxicity: when tissues overeat. Curr Opin Lipidol. 2003;281–7.
45. Yoshino J, Mills KF, Yoon MJ, Imai S ichiro. Nicotinamide Mononucleotide, a Key NAD+ Intermediate, Treats the Pathophysiology of Diet- and Age-Induced Diabetes in Mice. Cell Metab [Internet]. 2011 Oct;14(4):528–36. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1550413111003469
46. Meireles M, Rodríguez-Alcalá LM, Marques C, Norberto S, Freitas J, Fernandes I, et al. Effect of chronic consumption of blackberry extract on high-fat induced obesity in rats and its correlation with metabolic and brain outcomes. Food Funct. 2016;7(1):127–39.
47. Zhang XY, Guo CC, Yu YX, Xie L, Chang CQ. Establishment of high-fat diet-induced obesity and insulin resistance model in rats. Beijing da xue xue bao Yi xue ban = Journal of Peking University Health sciences. 2020;52(3):557–63.
48. Schwingshackl L, Hoffmann G. Comparison of Effects of Long-Term Low-Fat vs High-Fat Diets on Blood Lipid Levels in Overweight orObese Patients: A Systematic Review and Meta-Analysis. J Acad Nutr Diet [Internet]. 2013;113(12):1640–61. Available from: http://dx.doi.org/10.1016/j.jand.2013.07.010
49. Tan BL, Norhaizan ME. Effect of High-Fat Diets on Oxidative Stress, Cellular Inflammatory Response and Cognitive Function. Nutrients [Internet]. 2019 Oct 25;11(11):2579. Available from: https://www.mdpi.com/2072-6643/11/11/2579
50. Buijsse B, Feskens EJM, Schulze MB, Forouhi NG, Wareham NJ, Sharp S, et al. Fruit and vegetable intakes and subsequent changes in body weight in European populations: Results from the project on Diet, Obesity, and Genes (DiOGenes). American Journal of Clinical Nutrition. 2009;90(1):202–9.
51. González-Castejón M, Rodriguez-Casado A. Dietary phytochemicals and their potential effects on obesity: A review. Pharmacol Res. 2011;64(5):438–55.
52. Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, et al. Flavonoid intake and risk of chronic diseases. American Journal of Clinical Nutrition. 2002;76(3):560–8.
53. Montonen J, Järvinen R, Heliövaara M, Reunanen A, Aromaa A, Knekt P. Food consumption and the incidence of type II diabetes mellitus. Eur J Clin Nutr. 2005;59(3):441–8.
54. Wedick NM, Pan A, Cassidy A, Rimm EB, Sampson L, Rosner B, et al. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. American Journal of Clinical Nutrition. 2012;95(4):925–33.
55. Carter P, Gray LJ, Troughton J, Khunti K, Davies MJ. Fruit and vegetable intake and incidence of type 2 diabetes mellitus: Systematic review and meta-analysis. BMJ (Online). 2010;341(7772):543.
56. Villegas R, Xiao OS, Gao YT, Yang G, Elasy T, Li H, et al. Vegetable but not fruit consumption reduces the risk of type 2 diabetes in Chinese women. Journal of Nutrition. 2008;138(3):574–80.

57. Cooper AJ, Khaw KT, Sharp SJ, Wareham NJ, Lentjes MAH, Forouhi NG, et al. A prospective study of the association between quantity and variety of fruit and vegetable intake and incident type 2 diabetes. Diabetes Care. 2012;35(6):1293–300.
58. Liu S, Serdula M, Janket SJ, Cook NR, Sesso HD, Willett WC, et al. A prospective study of fruit and vegetable intake and the risk of type 2 diabetes in women. Diabetes Care. 2004;27(12):2993–6.
59. Belwal T, Nabavi S, Nabavi S, Habtemariam S. Dietary Anthocyanins and Insulin Resistance: When Food Becomes a Medicine. Nutrients [Internet]. 2017 Oct 12;9(10):1111. Available from: http://www.mdpi.com/2072-6643/9/10/1111
60. Sasaki R, Nishimura N, Hoshino H, Isa Y, Kadowaki M, Ichi T, et al. Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to downregulation of retinol binding protein 4 expression in diabetic mice. Biochem Pharmacol. 2007;74(11):1619–27.
61. Norberto S, Silva S, Meireles M, Faria A, Pintado M, Calhau C. Blueberry anthocyanins in health promotion: A metabolic overview. J Funct Foods. 2013;5(4):1518–28.
62. Meireles M, Marques C, Norberto S, Fernandes I, Mateus N, Rendeiro C, et al. The impact of chronic blackberry intake on the neuroinflammatory status of rats fed a standard or high-fat diet. Journal of Nutritional Biochemistry [Internet]. 2015;26(11):1166–73. Available from: http://dx.doi.org/10.1016/j.jnutbio.2015.05.008
63. DeFuria J, Bennett G, Strissel KJ, Perfield JW, Milbury PE, Greenberg AS, et al. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. Journal of Nutrition. 2009;139(8):1510–6.
64. Törrönen R, Kolehmainen M, Sarkkinen E, Mykkänen H, Niskanen L. Postprandial glucose, insulin, and free fatty acid responses to sucrose consumed with blackcurrants and lingonberries in healthy women. American Journal of Clinical Nutrition. 2012;96(3):527–33.
65. Törrönen 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.
66. Sivamaruthi BS, Kesika P, Chaiyasut C. The influence of supplementation of anthocyanins on obesity-associated comorbidities: A concise review. Vol. 9, Foods. MDPI Multidisciplinary Digital Publishing Institute; 2020.
67. Lee YM, Yoon Y, Yoon H, Park HM, Song S, Yeum KJ. Dietary anthocyanins against obesity and inflammation. Vol. 9, Nutrients. MDPI AG; 2017.
68. Stull AJ, Cash KC, Johnson WD, Champagne CM, Cefalu WT. Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. Journal of Nutrition. 2010;140(10):1764–8.
69. Yang L, Qiu Y, Ling W, Liu Z, Yang L, Wang C, et al. Anthocyanins regulate serum adipsin and visfatin in patients with prediabetes or newly diagnosed diabetes: a randomized controlled trial. Eur J Nutr [Internet]. 2021;60(4):1935–44. Available from: https://doi.org/10.1007/s00394-020-02379-x
70. Berndt J, Klöting N, Kralisch S, Kovacs P, Fasshauer M, Schön MR, et al. Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. Diabetes. 2005;54(10):2911–6.
71. Haider DG, Holzer G, Schaller G, Weghuber D, Widhalm K, Wagner O, et al. The adipokine visfatin is markedly elevated in obese children. J Pediatr Gastroenterol Nutr. 2006;43(4):548–9.
72. Owczarek AJ, Olszanecka-Glinianowicz M, Kocełak P, Bożentowicz-Wikarek M, Brzozowska A, Mossakowska M, et al. The relationship between circulating visfatin/nicotinamide phosphoribosyltransferase, obesity, inflammation and lipids profile in elderly population, determined by structural equation modeling. Scand J Clin Lab Invest [Internet]. 2016 Nov 16;76(8):632–40. Available from: https://www.tandfonline.com/doi/full/10.1080/00365513.2016.1230884
73. Kieswich J, Sayers SR, Silvestre MF, Harwood SM, Yaqoob MM, Caton PW. Monomeric eNAMPT in the development of experimental diabetes in mice: a potential target for type 2 diabetes treatment. Diabetologia [Internet]. 2016;59(11):2477–86. Available from: http://dx.doi.org/10.1007/s00125-016-4076-3

74. Caton PW, Kieswich J, Yaqoob MM, Holness MJ, Sugden MC. Nicotinamide mononucleotide protects against pro-inflammatory cytokine-mediated impairment of mouse islet function. Diabetologia. 2011;54(12):3083–92.
75. Istek N, Gurbuz O. Investigation of the impact of blueberries on metabolic factors influencing health. J Funct Foods. 2017 Nov 1;38:298–307.
76. Bhaswant M, Brown L, Mathai ML. Queen Garnet plum juice and raspberry cordial in mildly hypertensive obese or overweight subjects: A randomized, double-blind study. J Funct Foods. 2019 May 1;56:119–26.

APPENDIX 1 - Protein Quantification



Ovalbumin concentration (mg/mL)

Samples	Fluorescence 450/618	Sample - Control	Concentration mg/ml	Mean concentration ug/ml
CN 1	174596	245503,00		
CN 2	316410			
1A	8267713	8022210,00	0,674292648	740,1128538
1B	9456416	9210913,00	0,774206973	
1C	9428243	9182740,00	0,771838941	
2A	17138964	16893461,00	1,419949933	1454,585242
2B	18229482	17983979,00	1,511611491	
2C	17284638	17039135,00	1,432194303	
3A	10039976	9794473,00	0,823257074	782,3288398
3B	9327044	9081541,00	0,763332838	
3C	9292111	9046608,00	0,760396607	
4A	724820	479317,00	0,040288141	44,8427895
4B	768796	523293,00	0,043984466	
4C	843407	597904,00	0,050255761	
5A	8088578	7843075,00	0,659235773	694,5808558
5B	9361685	9116182,00	0,766244526	
5C	8076996	7831493,00	0,658262268	
6A	11436470	11190967,00	0,940636903	943,9925323
6B	10913128	10667625,00	0,896648319	
6C	12079580	11834077,00	0,994692375	
7A	18635964	18390461,00	1,545777615	1552,974085
7B	18801382	18555879,00	1,559681532	
7C	18727400	18481897,00	1,553463106	
8A	14532798	14287295,00	1,200893267	1144,418968
8B	13265298	13019795,00	1,094355801	
8C	13784636	13539133,00	1,138007836	
9A	12958072	12712569,00	1,068532463	1076,431786
9B	12832597	12587094,00	1,057985885	
9C	13365487	13119984,00	1,102777009	
10A	15576896	15331393,00	1,288653075	1243,121357
10B	15020779	14775276,00	1,241909646	
10C	14507910	14262407,00	1,198801351	
11A	12370601	12125098,00	1,019153629	1074,98282
11B	13308773	13063270,00	1,098010015	
11C	13425066	13179563,00	1,107784817	
12A	15304162	15058659,00	1,265728902	1230,045252
12B	14677459	14431956,00	1,213052491	
12C	14657256	14411753,00	1,211354364	
13A	10152386	9906883,00	0,832705498	795,4746807
13B	9169969	8924466,00	0,750130177	
13C	9805973	9560470,00	0,803588367	
14A	9796456	9550953,00	0,802788432	760,8432377
14B	9489265	9243762,00	0,776968037	
14C	8606553	8361050,00	0,702773244	
15A	10342871	10097368,00	0,848716377	773,3350603
15B	8938529	8693026,00	0,730676898	
15C	9056728	8811225,00	0,740611906	
16A	8017003	7771500,00	0,653219663	652,2643982
16B	8566884	8321381,00	0,699438936	
16C	7433027	7187524,00	0,604134595	
17A	8931344	8685841,00	0,730072976	733,7779581
17B	9091748	8846245,00	0,74355545	
17C	8903177	8657674,00	0,727705449	
18A	12363966	12118463,00	1,018595936	1022,567759
18B	13359197	13113694,00	1,102248315	
18C	11510496	11264993,00	0,946859028	
19A	4959730	4714227,00	0,396245998	354,0138176
19B	4404290	4158787,00	0,349559473	
19C	4007833	3762330,00	0,316235982	
20A	7153023	6907520,00	0,580599355	596,5728305
20B	7310494	7064991,00	0,593835301	
20C	7565672	7320169,00	0,615283836	
21A	5470257	5224754,00	0,43915744	472,2621685
21B	5458367	5212864,00	0,438158047	
21C	6663710	6418207,00	0,539471018	
22A	8086443	7840940,00	0,659056319	626,0456467
22B	7991632	7746129,00	0,651087149	
22C	7003048	6757545,00	0,567993472	
23A	13191334	12945831,00	1,088138888	1018,925817
23B	12196117	11950614,00	1,004487686	
23C	11716221	11470718,00	0,964150878	
24A	6789284	6543781,00	0,550025918	641,18198
24B	9153666	8908163,00	0,748759858	
24C	7678414	7432911,00	0,624760165	

APPENDIX 2 – Western Blots