Propionate

Al-Lahham, Sa'ad Hussein Mustafa

Propionate: a Candidate Metabolite to Link Colonic Metabolism to Human Adipose Tissue Inflammation

Sa’ad H. M. Al-Lahham

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Propionate: a Candidate Metabolite to Link Colonic Metabolism to Human Adipose Tissue Inflammation

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Sa'ad Hussein Mustafa Al-Lahham

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Promotor:	Prof. dr. R. J. Vonk
Copromotor:	Dr. K. Venema
Beoordelingscommissie:	Prof. dr. D. Hoekstra Prof. dr. J. M. van Dijl Prof. dr. H. Moshage

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To my parents

Paranimfen:	Margien Raven
	Diederik Esser

Contents

Chapter 1

General Introduction and Scope of the Thesis

1.1 General introduction

The prevalence of obesity is increasing worldwide, especially in the US, where it has been reported very recently that the prevalence of obesity and overweight in 2007-2008 was 68% (1); in the Netherlands it was 51% in 2004 (2). Obesity is detrimental to the quality of life and implies high health costs as a consequence of its associated low-grade inflammation (3), which is implicated in the pathogenesis of obesity-associated diseases, including insulin resistance, diabetes (4) and cardiovascular diseases (5;6).

1.2 Etiology of obesity

The etiology of obesity, and consequently its associated diseases, is complex and involves biological and life style factors. An important biological factor that contributes to the development of obesity and its associated pathologies is the white adipose tissue as we will discuss later. The role of life style factors, including nutrition, is poorly understood. Regular intake of energy-dense foods and low physical activity are associated with the development of obesity. Recently, light was shed on an interesting metabolic factor, the colonic metabolism, as we will discuss in more details below, which could have important impact for our dietary recommendations.

1.2.1 White adipose tissue

White adipose tissue plays a central role in the development of obesity. It is the largest organ in the human body where excessive triacylglycerol is stored. In

addition, it is an essential endocrine organ that produces various adipokines, which are actively involved in the regulation of the whole body homeostasis by influencing a variety of biological and physiological processes, including food intake, regulation of energy balance, insulin action, lipolysis and lipogenesis. During obesity, these processes are dysregulated; leading to an increased production of various inflammatory parameters, making adipose tissue the primary site of obesity induced chronic inflammation.

Adipose tissue is composed of various cell types including adipocytes and macrophages, which both are involved in the induction of inflammation. Macrophages infiltration into human adipose tissue was shown to be increased during obesity (7).

1.2.2 Microbiota

Recently, it has become clear that colonic metabolism is an important factor in the contribution to the development of obesity. Both the composition of the colonic microbiota and diet are important in this respect. Gordon and colleagues have shown that the intestinal bacteria in obese humans and mice differ from those in lean individuals. Furthermore, colonization of germ-free mice with microbiota from obese mice results in a significantly greater increase in total body fat than colonization with microbiota from lean mice (8;9). Conversely, the majority of the studies show that dietary fiber increases post-meal satiety, decreases body weight, fat mass and the severity of diabetes in humans and mice (10-15). The mechanisms by which the intestinal microbiota composition and metabolism can have such consequences on obesity-related pathophysiology remain, however, obscure.

1.3 Could propionic acid constitute the link between microbiota and obesity?

Fermentation of dietary fiber by the colonic microbiota is the primary source for the production of short-chain fatty acids (SCFA), in particular acetic acid, butyric acid, and propionic acid (PA). SCFA have recently attracted considerable attention, because of their impact on the host's health. However, most of the studies investigated the roles of butyrate alone or mixed with other SCFA in colonic health and most of the few studies in which PA was considered alone were investigated in ruminants. Although 90% of butyric acid is utilized by colonocytes and very little reaches the visceral tissues (16), several peripheral effects of butyrate were described. The majority of the PA quantity produced in the colon (20 mmol/kg) (17) is absorbed, passes the colonocytes and the viscera, and drains into the portal vein. PA can be partially metabolized by the colonocytes; the quantity that is utilized by visceral tissues, e.g. omental adipose tissue, has not been examined yet. The quantity of PA in the portal vein in non-fasting humans was demonstrated to be approximately 0.1 mM (17-19). Together, it is fair to say that PA is a significant microbiota-derived metabolite for the analyses of its interaction with the host physiology.

1.4 The underlying molecular mechanisms that mediates PA signal

In 2003, three independent studies found that PA was the most potent and efficacious ligand for GPCR41, and equipotent with acetate for GPCR43 (20-22). GPCR41 and GPCR43, like other GPCRs, are linked to GTP-binding proteins (G-proteins). G-proteins are attached to the cytoplasmic face of the receptor, where

they serve as relay molecules functionally coupling the receptors to their downstream targets. G-proteins are classified into four major classes, namely Gs, Gi/o, Gq/11 and G12/13. Each of them is specific for a particular set of GPCRs and a particular set of downstream targets. GPCR41 was shown to initiate its signaling through coupling with the Gi/o family of G-proteins (20;21). On the other hand, GPCR43 was shown to be coupled to both Gi/o and Gq families of G-proteins (20-22). GPCR43 was found to be expressed at high levels and GPCR41 was expressed to a lesser extent in various immune cells (21). In addition, both of these receptors were shown to be present on adipose tissue (20). Taken together, these receptors provide an important link in the microbial PA production, adipose tissue and inflammation triangle.

1.5 Hypothesis

Taken altogether, one could envision, as illustrated in figure 1, that PA could constitute the elusive link between colonic-microbiota metabolism and the biology of human adipose tissue including inflammation, insulin sensitivity, lipogenesis and adipogenesis, and consequently the whole body homeostasis.

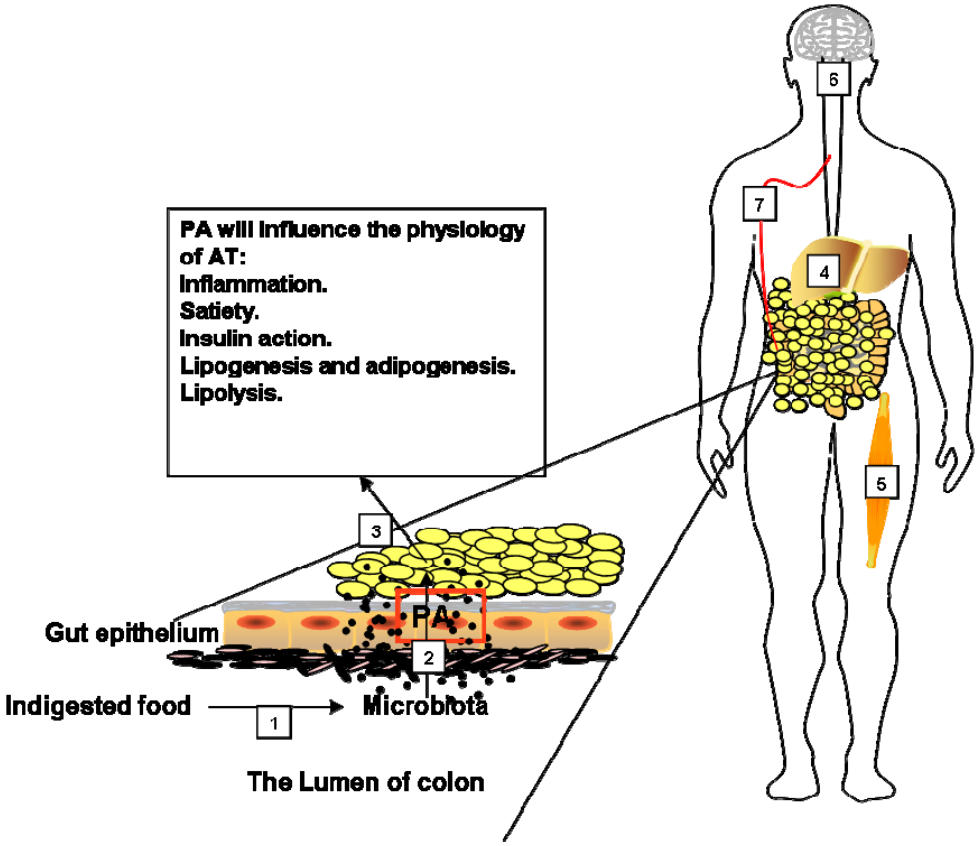

Figure 1: Schematic representation of our hypothesis. Undigested food reaches the colon where it is fermented by the microbiota (1). PA is a primary product of this fermentation (2). PA passes the gut epithelium and interacts with adipose tissue (3). It influences the physiology of adipose tissue (including inflammation, satiety, insulin action, lipogenesis, adipogenesis and lipolysis) and consequently the whole body homeostasis, e.g. in liver (4), muscle tissue (5) and central (6) and peripheral (7) nervous system.

1.6 Scope of the thesis

Adipose tissue is a primary site of obesity-induced inflammation which is emerging as an important contributor to obesity-related diseases. The factors influencing adipose tissue-induced inflammation remain poorly understood. However, dietary fiber diets appear to be protective to obesity-related diseases, suggesting a cross-talk between the colonic metabolism and adipose tissue-induced inflammation. Short-chain fatty acids, e.g. propionic acid (PA), are one of the principal products of the dietary fiber fermentation by microbiota. This leads us to hypothesize that PA could constitute the elusive link between colonic-microbiota metabolism and the physiology of human adipose tissue. To test this hypothesis we formulated the following research aims, after reviewing the recent knowledge about metabolic, immunological and potential adverse effects of PA:

1. To investigate the influence of PA on the production of various markers of physiologically relevant functions by human adipose tissue *ex vivo,* including adipokines, cytokines and chemokines.
2. To determine the contribution of the adipocytes and macrophages to the observed anti-inflammatory effects of PA.
3. To unravel the role of Gi/o-protein in mediating the effects of PA in human adipose tissue explants.

To achieve our aims, the following studies have been conducted.

1.6.1 Summary of various aspects of PA

PA has long been underestimated in terms of its physiological impact. Most studies addressed the effects of other short chain fatty acids, i.e. butyrate and to a lesser

extent acetate. Therefore, we made the first review article to discuss various aspects of PA in humans and animals, drawing the attention to the recent findings and the importance of PA for the health of the host. It has been reported that PA is produced by the fermentation of indigested food by the microbiota in the colon, which can reach the blood compartment where it reduces (the long chain) fatty acids levels and their production in plasma and liver. In addition, it has been reported that PA reduces food intake in animals. Although it exerts pronounced immunosuppressive actions; however so far no study investigated the influence of PA on human adipose tissue inflammation, which is the primary site of obesity induced inflammation. Therefore, we conducted the studies as discussed below. **(Chapter-2).**

1.6.2 PA influence on the production of adipokines and the involvement of Gi/o-PCR in their responses

We analysed the effect of PA on the production of leptin, resistin and adiponectin by women adipose tissue *ex vivo*, to predict its effect on satiety, pro-inflammation and insulin sensitivity, respectively. Leptin production was stimulated in adipose tissue by PA treatment, whereas resistin was strongly inhibited and adiponectin was not influenced. This may indicate that PA reduces food intake and increases energy expenditure (through leptin induction) and it may possess anti-inflammatory properties (through resistin inhibition). Subsequently we investigated the role of Gi/o-proteins in mediating the effects of PA on these adipokines. The response of leptin and resistin was mediated via Gi/o-dependent and independent pathways, respectively. **(chapter-3)**.

1.6.3 PA modulates adipose tissue inflammation via Gi/o-dependent and independent pathways

The inhibitory effect of PA on the production of the pro-inflammatory adipokine (resistin), which we observed in chapter-2, persuaded us to further examine the effect of PA on the inflammation of adipose tissue *ex vivo*. It was revealed that PA suppressed the production of various cytokines, chemokines and the ATM markers from adipose tissue also via Gi/o-dependent and independent pathways. In addition, ATM markers were either not detected or very low in adipocytes compared to adipose tissue. Taken together, this implies that non-adipocyte cells, most likely ATM, contribute to the immunomodulatory effects of PA. The anti-inflammatory influence of PA on the adipose compartment may provide a mechanistic explanation to the preventive influence of diets leading to the increased microbial production of such SCFA on the development of metabolic syndrome and indeed we found that PA positively influences the production of the markers related to insulin sensitivity, lipogenesis and adipogenesis. (**chapter-4**).

1.6.4 PA exerts anti-inflammatory effects on human macrophages

To evaluate further the role of macrophages, the effect of PA on the secretome of human macrophages was investigated via a proteomics approach, SILAC, and validated by multiplex-ELISA assay. Again, PA showed overall anti-inflammatory effects, which was demonstrated by the significant inhibition on the release of 17 inflammatory proteins. (**chapter-5**).

1.6.5 Human adipocytes exhibit an immune cell like behavior

Our studies so far suggest that adipocytes do not contribute to the effects of PA. However, after studying human preadypocytes and adipocytes in depth, we found that these cells exhibit an immune cell like behavior. It was shown, by genomics and proteomics approaches, that both preadipocytes and adipocytes produce various inflammatory parameters. Interestingly, adipocytes were shown to behave as immune cells when they were treated with LPS (induction and reduction of pro-inflammatory and anti-inflammatory parameters, respectively). Finally, we examined whether adipocytes are involved in the initiation of the inflammation process and we demonstrated that LPS-stimulated adipocytes indeed recruited more CD+4 T-cells. (**chapter-6**).

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Chapter 2

Biological Effects of Propionic Acid in Humans; Metabolism, Potential Applications and Underlying Mechanisms

Sa'ad H. Al-Lahham, Maikel P. Peppelenbosch, Han Roelofsen, Roel J. Vonk and Koen Venema

BBA-Molecular and Cell Biology of Lipids (conditionally accepted)

Abstract

Undigested food is fermented in the colon by the microbiota and gives rise to various microbial metabolites. Short-chain fatty acids (SCFA), including acetic, propionic and butyric acid, are the principal metabolites produced. However, most of the literature focuses on butyrate and to a lesser extent on acetate; consequently, potential effects of propionic acid (PA) on physiology and pathology have long been underestimated. It has been demonstrated that PA lowers fatty acids content in liver and plasma, reduces food intake, exerts immunosuppressive actions and probably improves tissue insulin sensitivity. Thus increased production of PA by the microbiota might be considered beneficial in the context of prevention of obesity and diabetes type 2. The molecular mechanisms by which PA may exert this plethora of physiological effects are slowly being elucidated and include intestinal cyclooxygenase enzyme, the G-protein coupled receptors 41 and 43 and activation of the peroxisome proliferator-activated receptor γ, in turn inhibiting the sentinel transcription factor NF-κB and thus increasing the threshold for inflammatory responses in general. Taken together, PA emerges as a major mediator in the link between nutrition, gut microbiota and physiology.

2.1 Introduction

The link between dietary intake and physiology is long-recognized as evident from the age-old adage “you are what you eat” and other colloquial –but not entirely unsupported- expressions like “Feed a cold, starve a fever” high light the recognition within the general population of the connection between nutrition and pathology (1). Indeed many investigators now assume that environmental factors, e.g. dietary patterns, are as important as the genetic makeup in the contribution for the phenotypes of individuals, especially the propensity for disease. Especially for so-called prebiotic diets in general and long chain O-linked oligofructoses (fructans) in particular (2), which are associated with general better health, and indeed generation of genetically modified crops capable of producing large quantities of fructans has become an industry by it self (3). Although many of the molecular and immunological aspects by which dietary components could influence physiology (4) or even pathology (5) have been uncovered it is fair to say that the exact mechanism by which nutritional modification of metabolism of the microbiota interacts with the host is still largely obscure at best (6). Here we argue that propionic acid is an important link in the nutrition, microbiome and physiology triangle.

A large body of research indicates that dietary fiber has a profound effect on general health. These include the increase of post-meal satiety and the decrease of body weight, fat mass and the severity of diabetes (7-12). These effects may be contributed via the fermentation of dietary fiber by the colonic microbiota and in turn the production of various metabolites, such as SCFA, which are absorbed by the host and influence its energy homeostasis (8, 13). The microbiota also influences the development of obesity and its associated diseases (14). This influence depends on microbiota composition within an individual, which seems to

be defined via a combination of environmental and genetic factors that could favor either obese or lean phenotype (15, 16).

Fermentation of dietary fiber by the colonic microbiota is the primary source for the production of SCFA, i.e. acetic, propionic and butyric acid (figure 1). SCFA have recently attracted considerable interest, because of their possible importance for host health. Most of the studies (and reviews) on the interaction of SCFA and mammalian physiology, however, concentrate either solely on the role of butyrate alone (17), or on the effects of complex SCFA mixtures, PA mainly being investigated in the context of ruminant physiology in general, and on its role in liver physiology and metabolism in particular. Although in ruminants PA and other SCFA are the major source of energy (PA is the primary precursor for glucose production in ruminants), whereas glucose is the major source for humans, there is good evidence, as we discuss below, that PA is an important factor in human physiology as well.

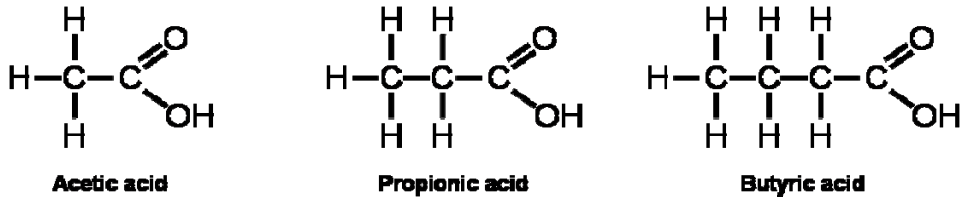

Figure 1: The molecular structure of the short chain fatty acids, acetic, propionic and butyric acid.

2.2 Propionic acid metabolism

2.2.1 Propionic acid occurrence and production

PA occurs naturally in a few food products; for example PA is present in low quantities in milk and relatively higher levels in diary products such as yogurt and

cheese, obviously due to bacterial fermentation, mostly by propionibacteria (18, 19). It is available also as a preservative (E280) in food products, since it has anti-fungal and anti-bacterial effects (20, 21). These food-sources however, do not lead to significant amounts of PA in the human circulation as quantities involved pale in comparison to the primary natural source for PA in humans, which is derived from the fermentation of undigested food by the colonic microbiota (22). In the colon, PA is produced by fermentation of polysaccharides, oligosaccharides, long-chain fatty acids, protein, peptides and glycoprotein precursors by the anaerobic colonic microbiota (figure 2) (23), although in quantitative terms indigested carbohydrates, such as dietary fiber and resistant starch, represents the major source for PA production. These substrates are mainly composed of hexoses and pentoses, which are fermented by the microbiota through a variety of pathways. Hexoses are broken down mainly via the glycolytic pathway or they are converted to 6-phospho-gluconate and then metabolized via the pentose phosphate pathway, the same pathway through which pentoses are metabolized. Pyruvate is the principal metabolite of these fermentation reactions; however very little pyruvate is found in the colon, because it is converted to a series of end products, such as PA and other SCFA. PA is produced from pyruvate via two main pathways: i) Succinate decarboxylation pathway, in which CO_2 is fixed to pyruvate to form succinate, which is subsequently decarboxylated to propionate; and ii) Acrylate pathway, in which PA is produced from acrylate with lactate as a precursor (24, 25).

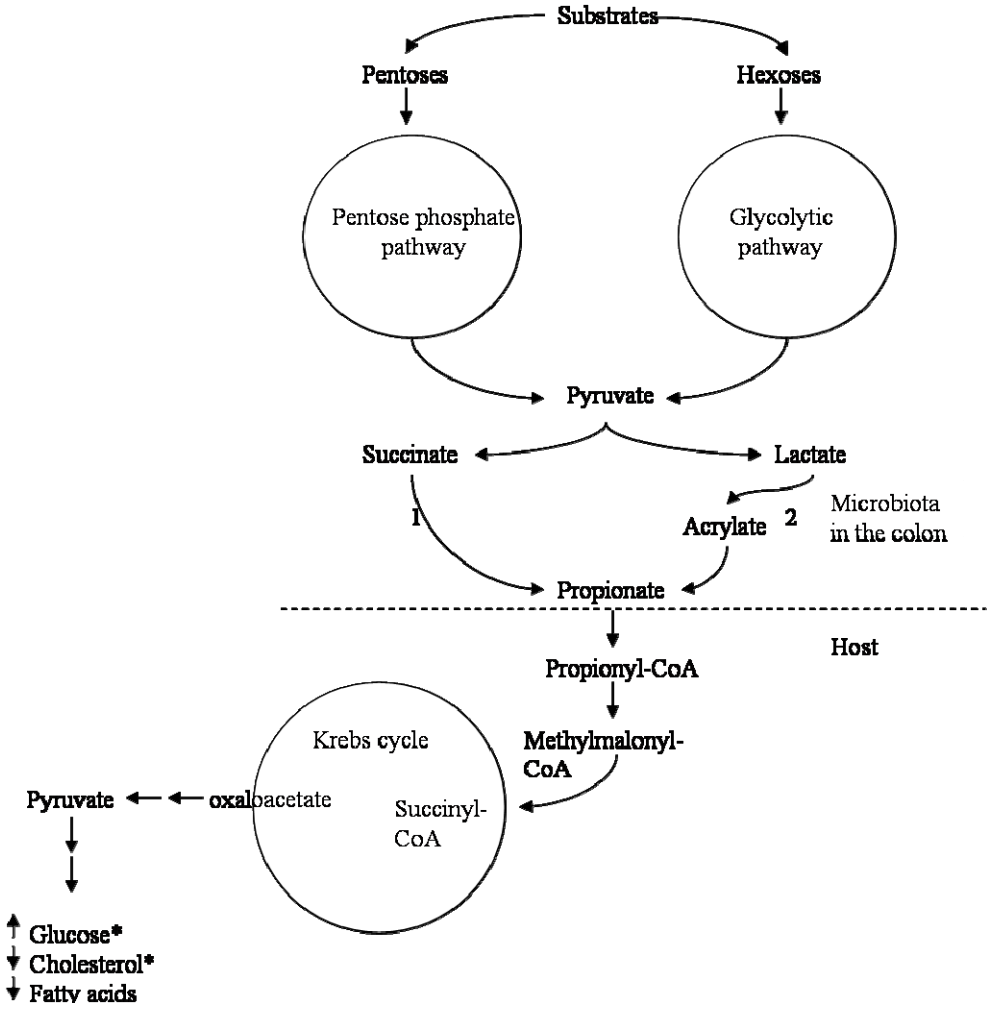

Figure 2: PA metabolism. Undigested food that reaches the colon is broken down by the microbiota into hexoses and pentoses molecules, which are further metabolized into pyruvate. Pyruvate is converted into PA via (1) Succinate Decarboxylation or (2) Acrylate pathways. PA is absorbed by the host, where it induces the production of glucose and suppresses the production of fatty acids and cholesterol.*; the effects of PA on the production of glucose and cholesterol in humans are controversial.

Despite the technical and ethical issues involved, a few studies measured the quantities of PA and other SCFA in human colon and portal vein. The PA quantity in the human colon was reported to be 20 mmol/Kg and to depend on the

balance between production and absorption (22, 26), the type of microbiota, the quantity and the type of the substrate and the gut transit time (24). In contrast to butyrate, the majority of the PA produced in the colon is absorbed, passes the colonocytes and the viscera, and drains into the portal vein. PA can be partially metabolized by colonocytes; however, the quantity that is utilized by visceral tissues, e.g. visceral adipose tissue, has not been examined yet. The quantity of PA in the portal vein in non-fasting humans was demonstrated to be approximately 0.1 mM; while it was 3-fold lower in blood derived from fasting humans (22, 27, 28). Around 90% of PA quantity is metabolized by the liver and the rest is transported into peripheral blood (24), where its quantity in humans was reported 6 μM (27, 29-31), far in excess of that of butyrate, but lower than acetate. High PA quantities also occurred in certain pathological situations, such as gingival inflammation and propionic acidemia (32, 33). Together, it is fair to say that propionic acid is likely to interact with host physiology.

2.2.2 Propionic acid and glucose metabolism

PA metabolism was extensively studied in ruminants where it is a major glucose precursor (34-36). Briefly, PA is converted to propionyl-CoA and enters the Krebs cycle at the level of succinyl-CoA (figure 2). This leads to oxaloacetate elevation, from which a large amount, in the liver, is converted to glucose. PA is the main source for glucose production from SCFA, while cellular biochemistry does not allow acetate, butyrate and longer chain fatty acids to contribute to net synthesis of glucose. The only pathway for fatty acids with even numbers of carbon atoms is via acetyl-CoA and Krebs cycle. When acetyl-CoA enters the cycle the 2 carbon atoms are lost as CO_2, so no net gain of oxaloacetate occurs (36). Biotin and vitamin B12 are essential factors for the anabolic effect (37). This suggests that reducing biotin and vitamin B12 increases the PA quantity as under these

conditions PA is no longer converted to glucose and consequently deficiency in these factors lead to reduced energy uptake of the host organism. Indeed, in severe B12 deficiency in sheep, a reduction in feed intake and an elevation of the PA quantity in blood was found (38). Much less is known about the effect of PA on glucose production in humans (which anyway rely on other sources to maintain glucose levels), and existing data are inconsistent (24, 39, 40).

2.2.3 Propionic acid and cholesterol/fatty acid metabolism

Diets supplemented with PA exhibit hypocholesterolemic effects in animals (41, 42); however, in humans, the effect of PA on cholesterol levels was inconclusive (24). Recently, evidence has been accumulating that PA has fatty acid-lowering effects. In animals, PA decreased fatty acid production and quantity in the liver (43, 44) and in plasma (41, 45). It seems that the effect of PA on fatty acid levels in humans is similar to that in animals. Apart from inhibiting the production of fatty acids in liver, the observed decreases in fatty acid levels by PA probably also is derived from inhibition of lipolysis and the induction of adipogenesis in adipose cells and tissue *in vitro* (46, 47) and *in vivo* data showed that PA receptor (GPCR41)-deficient mice exhibited less adiposity compared to the wild type mice (48). Moreover, leptin, which is an adiposity marker, was induced by PA treatment of human and mouse adipose tissue (49, 50). In view of the established role of circulating fatty acids in vascular pathology, these effects may well contribute to the beneficial effects of diets associated with increased PA-production.

2.3 Propionic acid's physiological roles and potential applications

2.3.1 Inflammation

It is now well established that the gastrointestinal tract is permanently in a state of low grade inflammation (51). Dietary fiber intake, which is the primary substrate for PA production, has been associated with a reduction in low grade inflammation (8) and in intestinal inflammatory pathogenesis (52-54). PA, as we mentioned earlier, has anti-fungal and -bacterial effects (55). Moreover, PA has moderate inhibitory activity on cyclooxygenase (56), a major enzyme in the production of pro-inflammatory eicosanoids (57). *In vitro* studies have provided considerable evidence that PA has anti-inflammatory properties, apart from its influence on eicosanoid metabolism. PA possesses anti-microbial activity against the colonization of the gastrointestinal tract by pathogenic bacteria such as *Salmonella* (21), via, for example, the inhibition of the expression of the invasion genes in *Salmonella typhimurium* (58), which are essential to invade and penetrate the intestinal epithelium. However, it seems that the inhibition of inflammation by PA depends on several factors, such as its concentration, the pH of the extracellular *milieu* and the reason of inflammation, as we describe below.

The proliferation of human and animal lymphocytes activated by a mitogen is inhibited by PA treatment (59, 60). In contrast, Cavaglieri et al reported that PA did not influence the proliferation of activated lymphocytes (61). This discrepancy might be a consequence of the PA concentration employed in the various studies; while Cavaglieri et al used a 2 mM concentration, Wajner et al (60) demonstrated that PA concentrations above 2.5 mM were required to reduce the proliferation of activated lymphocytes. In addition, Curi et al (59) found that

PA concentrations equal to or more than 3 mM showed a remarkable high inhibition of lymphocyte proliferation, whereas 2 mM produced only a mild reduction in lymphocyte proliferation. Likewise, we have shown (49) that 3 mM PA or higher inhibited the production of the pro-inflammatory cytokine, resistin, by human adipose tissue. Thus at high concentrations PA can directly inhibit the adaptive lymphocyte compartment, but comparison to the PA concentrations present in the portal and peripheral blood make the physiological relevance of these observations doubtful outside the visceral compartment itself.

Chemically speaking PA is an acid and because of its membrane permeable nature higher concentrations of PA may directly exert effects on host cell physiology by altering the intracellular pH. At bay with this notion is that the active cellular control of pH is pretty strict and that alternative modes of action seem more likely. It was a concern however in the studies of Brunkhorst et al (62) and Le Poul (63), who, when trying to demonstrate that PA-induced effects were mediated by activated G-proteins, included controls to show that the effects observed were not due to extracellular or cytoplasmic pH. Furthermore, in our own studies on PA biological activity (49), no evidence of a role for pH-modulation in PA effects was found. Conversely, however, there are good data that environmental pH determines whether or not PA can exert anti-inflammatory actions. Mills et al (64) demonstrated that PA at pH 5.5 inhibited the oxidative burst and phagocytosis of bovine neutrophils; while, at pH 6.7 it was stimulatory. As most physiological actions of PA can be expected to occur at a more alkaline pH, the physiological relevance of these anti-phagocytotic actions of PA is uncertain. Wajner et al (60) and Mills et al (64) indicated that the type of stimulant could influence the concentration of PA that is needed to suppress inflammation. In other words the cause of inflammation, e.g. bacteria, cytokines, adipokines, fatty acids and others can determine whether PA can exert its immunosuppressive properties, or not. This may clarify the inconsistency of neutrophils response to

PA. Indeed, Vinolo et al (65) showed that PA had no effect on phagocytic activity and ROS production in rat neutrophils and thus primary efficacy in the innate immune system is not targeted by PA, although subsequent changes in innate immune cell gene expression might occur: Tedelind et al (66) demonstrated that PA inhibited LPS-stimulated TNF-α release by human neutrophils. Moreover, a similar phenomenon was shown in endothelial cells; on the one hand Zapolska-Downar et al (67) demonstrated that PA inhibited the expression of adhesion molecules in cytokine-activated human endothelial cells. On the other hand Miller et al (68) found no influence of PA on non-activated endothelial cells and *in toto* a picture emerges that direct pathogen clearance by the innate immune system is not majorly influenced by PA (which might be in line with its beneficial rather than its adverse effects on inflammatory bowel disease, e.g. (69)). Effects in these cells are limited to modulation of gene expression.

In vivo, however, results obtained with SCFA effects on inflammatory diseases of the gut are somewhat disappointing, although there is no single study in which PA alone was investigated *in vivo*. Various studies revealed that enemas containing SCFA improved the clinical and inflammatory parameters of ulcerative colitis (70, 71). In contrast, other studies found only trends toward clinical improvement of ulcerative colitis (72, 73). The outcome of the intervention studies for diversion colitis was also inconclusive (74, 75). The equivocal results in human intervention studies may partly be explained by the differences in the setup of these studies, e.g. treatment duration, SCFA concentration and volume, and the small number of patients. Thus a convincing demonstration of efficacy of SCFA in treating these diseases has not been revealed and further studies are necessary to unravel this. Notably, these studies are not reflecting the effects of PA alone, and according to the *in vitro* studies, it is possible that enemas containing PA alone could improve the clinical and inflammatory parameters of gut inflammatory diseases. *In vivo*, the effects of PA on other inflammatory diseases, such as low grade inflammation, remain also unknown. However, *in vitro* we (unpublished

data) demonstrated that PA counteracts the inflammatory reaction in human adipose tissue, which is a primary source for obesity induced low grade inflammation.

2.3.2 Insulin sensitivity

Inhibitory effects of PA on free fatty acids metabolism and inflammation, which we mentioned earlier, suggest that PA could be a potential therapeutic agent to improve insulin sensitivity; since free fatty acid-elevation has been demonstrated to cause inflammation (76, 77) and vice versa (78, 79) and both lead to insulin resistance (79-83). We observed a PA-dependent increase in GLUT-4 in primary human adipose explants, which also point in this direction. Moreover, evidence has been described for induction of lipogenesis and adipogenesis and inhibition of lypolysis (46, 47). Furthermore, butyrate prevents and reverses diet-induced insulin resistance in mice (84). Taken together, good evidence exists that systemically relevant concentrations of PA might exert a beneficial effect on insulin sensitivity in the adipose compartment and thus PA may well form the elusive link between pre- and pro-biotic supplementation and its beneficial effects on obesity-related diseases.

2.3.3 Food intake and satiety

A substantial amount of evidence has shown that absorbed PA causes satiety and reduces food intake in ruminants (85-87). So far, in humans only two studies investigated the role of PA on satiety and both demonstrated that dietary supplementation of PA caused satiety (88, 89). The underlying mechanism remains

under debate. Studies in ruminants revealed that the mechanism by which PA conveys its effect on satiety is not simple and integrates neuronal, endocrine, paracrine and autocrine pathways between and within organs and tissues. One of the suggested mechanisms was hypertonia (90); however, further work (91) disproved that by showing that PA reduced feed intake more than equimolar acetate, mannitol, or saline. Anil and Forbes (86) reported in animal studies that receptors existed in the liver that were sensitive to PA and that surgical sectioning of the hepatic nerve plexus around the wall of the hepatic artery abolished the satiety effect of PA. Further splanchnic blockade with anaesthetic, splanchnotomy and hepatic vagotomy, as well as with total liver denervation, were shown to abolish the reduction in feed intake by portal infusion of PA, although as these procedures target innervation of the viscera *in toto*, other organs/tissues apart from the liver may be involved as well (92). Indeed vagal-innervated visceral adipose tissue is a primary endocrine tissue that produces adipokines, which regulate satiety and energy homeostasis. It was shown that PA induced the production of the satiety hormone leptin by human, mouse and ruminant adipose tissue (46, 49, 50). *In vivo*, in mice, PA administered via gavage induced leptin but did not reduce food intake. The latter was suggested to be due to gavage-induced stress (46), since in the same study, food intake was reduced in mice fed chow containing 3% of sodium propionate in diet as compared to controls fed equimolar sodium chloride. Leptin is a potent anorexigenic hormone that suppresses food intake through the central nervous system (93) and vagal neurons (94). This suggests that visceral adipose tissue is another potential mechanism to regulate the satiety induced by PA infusion and this notion would fit well with the direct effects of PA on the adipose tissues physiology, in particular with respect to release of adipokinetic hormones. Finally, food intake could be inhibited by adverse internal cues. For example, it was shown that the ingestion of a high quantity of PA by ruminants induced strong food aversions apparently due to nausea and discomfort (95-97).

2.4 Potential adverse effects

2.4.1 Propionic acidemia

High quantity of PA could also have potential adverse effects. This has been described in the metabolic disorder propinic acidemia,which is caused by defects in propionyl-CoA carboxylase enzyme (98). It is a biotin-dependent mitochondrial enzyme involved in the metabolism of odd-chain fatty acids and the amino acids methionine, threonine, isoleucine and valine by converting propionyl-CoA to methylmalonyl-CoA. This defect of the enzyme leads to increased amounts of PA and other acids and toxins such as ammonia, which causes food refusal, vomiting, immunosuppresion, mental retardation, predisposition for infections and sepsis (99, 100). However, (101) succeeded to improve these symptoms by liver transplantation, while minimal effects were shown on PA quantity. This suggests that these symptoms are not due to high PA quantity per sé, but rather due to accumulation of various metabolites. Another evidence to support this notion is derived from the fact that another organic acidemia (branched chain academia; (99)) causes the same symptoms, however without propionic acid accumulation (102, 103).

2.4.2 Autism spectrum disorders

Autism is a disorder of neural development including development deficiencies of language and social interaction skills, appearance of repetitive and disordered movements (104), hyperactivity, sensory disturbances, restricted interests and sometimes self injury (105, 106). Very recently and interestingly, it was

demonstrated that intraventricular infusions of PA caused behavioral and brain abnormalities in rats similar to those seen in humans suffering from autism via probably altering brain fatty acid metabolism (107-110). Rather large amounts have been used to induce the systems (e.g. 4 μl of 0.26 M solution). It therefore remains to be seen that the concentrations that are produced by the microbiota lead to similar effects.

2.4.3 Gingival inflammation

Gingival inflammation is an inflammatory process in the gum tissue of the mouth, which is caused by the release of short chain fatty acids in millimolar concentrations by periodontal bacteria. It was shown that PA is the major metabolite produced *in vitro* (by *Prevotella loescheii*) (111) and *in vivo* (112). In the *in vivo* study it was further shown that the subgingival concentration of PA was more than 10 fold higher (9.5 mM) in severely diseased patients than in mildly diseased patients (0.8 mM), while it was undetectable in the healthy sites of the mouth of the same patients. The same group demonstrated in a different study (113) that applying PA mixed with other short chain fatty acids into gingival margins of designated teeth induced gingival inflammation. The PA effect on gum tissue seems contradictory to the pronounced anti-inflammatory effects of PA that we discussed earlier; this suggests that PA effect on inflammation depends on the target tissue or type of inflammation.

2.5 Molecular mechanisms

2.5.1 Cyclooxygenase inhibition

As mentioned, PA has moderate inhibitory activity on cyclooxygenase (56), a major enzyme in the production of pro-inflammatory eicosanoids (57) and indeed several non steroidal anti-inflammatory drugs (NSAIDs), such as fenoprofen, flurbiprofen, ibuprofen and naproxen, are PA analogues (114). In view of the concentrations of PA in the colonic intestine, a direct anti-inflammatory effect of PA via cyclooxygenase is to be expected and this is a likely mechanism for the downregulation of low-grade mucosal inflammation by prebiotic diets. Furthermore, both prebiotic diets (2) and cyclooxygenase inhibition (115) are associated with reduced incidence of colorectal cancer and thus PA-dependent inhibition of cyclooxygenase may well be implicated in this effect as well. This notion is strongly supported by the observation of Comalada *et al*. (116) that bacterium-derived SCFA seem directly responsible for anti-carcinogenic effects of pre/pro-biotic supplementation in preclinical models of colon cancer. Thus, direct modulation of the colonic production of pro-inflammatory cycloocxygenase-derived eicosanoids may be an important factor by which PA suppresses intestinal immunology.

2.5.2 G-protein coupled receptors (GPCR)

In 2003, three independent studies found that PA was the most potent and efficacious ligand for GPCR41, and equipotent with acetate for GPCR43 (63, 117, 118). GPCR41 and GPCR43, like other GPCRs, are linked to GTP-binding

proteins (G-proteins). G-proteins are attached to the cytoplasmic face of the receptor, where they serve as relay molecules functionally coupling the receptors to their downstream targets. G-proteins are classified into four major classes, namely Gs, Gi/o, Gq/11 and G12/13. Each of them is specific for a particular set of GPCRs and a particular set of downstream targets. GPCR41 was shown to initiate its signaling through coupling with the Gi/o family of G-proteins, as evident from the absence of a response when transfected cells were treated with a Gi/o-protein-inhibitor (63, 117). On the other hand, GPCR43 was shown to be coupled to both Gi/o and Gq families of G-proteins (63, 117, 118). Notably, neither GCR41 nor GPCR43 has been tested for its ability to interact with a full panel of G proteins and families. GPCR43 was found to be expressed at high levels and GPCR41 was expressed to a lesser extent in various immune cells (117), providing an obvious link between the immune system and microbial PA production. Very recently Maslowski et al generated GPCR43 knockout mice and established the importance of GPCR43 as an anti-inflammatory chemoattractant receptor (119). In this study, GPCR43 knockout mice showed exacerbated inflammation in models of inflammatory diseases, i.e. colitis, arthritis and asthma. Wild type germ-free mice, which are devoid of bacteria and therefore produce little or no SCFA, showed a similar dysregulation of certain inflammatory responses. Furthermore, acetate supplementation in drinking water for germ free wild type mice produced a reduction in inflammation in the same disease models. As propionic acid is one of the two SCFA to reach significant concentrations in the circulation under normal conditions, these experiments strongly support the role of propionic acid production by the microbiota as an important anti-inflammatory mechanism.

Despite the dramatic phenotype of the GPRC43 KO, also GPCR41 might still turn out to be an important contributor to PA effects in physiology. Both receptors are expressed by human adipose tissue (46, 49, 50, 63, 117) leading to adipogenesis and inhibition of lipolysis in mouse adipocytes via GPCR43 (46, 47) while induction of leptin production, a marker for adiposity, occurs through

GPCR41 (50). *In vivo,* GPCR41 was responsible for gut microbiota induced-adiposity in mice (48). Thus the exact contribution of these two receptors to total PA functionality in the body requires further investigation.

2.5.3 PPARγ and NF-κB

The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) is a ligand activated transcription factor, in turn inhibiting the sentinel transcription factor, nuclear factor-kappa B (NF-κB) and thus increasing the threshold for inflammatory responses in general. Like chemically-related molecules (other fatty acids and anti-diabetic drugs, e.g. thiazolidinediones (120)), PA can activate PPARγ (our own unpublished results). PPARγ agonists were shown to inhibit the expression of genes regulated via NF-κB; this was through PPARγ-dependent and -independent mechanisms (121-124). PA down-regulated the activity of NF-κB in human colon adenocarcinoma cell line and endothelial cells (66, 67, 125), and direct interaction with PPARγ is thus a likely mechanism, providing yet another level in which immunity and PA may interact.

2.6 Conclusions and perspectives

PA has long been underestimated in terms of its physiological impact, most studies addressing the effects of butyrate and to a lesser extent acetate. Although the latter two are probably of principal importance in intestinal physiology, systemically they are less likely to have significant effects. PA is mainly produced by the fermentation of indigested food by the microbiota in the colon, but can reach the blood compartment and the adipose tissue, where it reduces fatty acids levels in

plasma via inhibition of lipolysis and induction of lipogenesis in adipose tissue and suppression of fatty acid production in liver. We speculate that this is a major mechanism by which prebiotics exert their effects on obesity related disease. Lowering plasma fatty acids may be especially important, since it is known that high plasma fatty acids cause inflammation. In addition, it is known that fatty acids and inflammatory factors cause insulin resistance (79-83), so the PA-lowering effects on fatty acids and inflammation might lead to the observed improvement in insulin sensitivity. These beneficial effects are usually associated with a reduction of body weight and indeed it has been demonstrated that PA inhibits food intake and increases the duration of satiety via the integrations of neuronal, endocrine, paracrine and autocrine pathways between and within organs and tissues. As depicted in figure 3, both adipose tissue and liver has been shown to be targets for PA. For example, PA influences the production of hormones by adipose tissue, such as the induction of leptin. This might contribute to the suppression of food intake, since leptin is a potent anorexigenic hormone that suppresses food intake through receptors expressed in the central nervous system (93) and vagal neurons, which innervates visceral adipose tissue. PA as mechanism for the interaction between microbiota and adipose tissue would provide a new paradigm in our view of humans as metaorganisms, and further research into this notion is certainly called for.

PA mediates its effects via various molecular mechanisms, which are now emerging. Directly, it influences pathogen physiology and down modulates intestinal cyclooxygenase activity, via the circulation it can stimulate its receptors GPCR41 and GPCR43 and anti-inflammatory effects via the associated signal transduction pathways. A last level is the inhibition of NF-κB through PPARγ. However, many questions remain. For example, why are there several molecular targets for PA and how do they interact with each others to convey PA signaling? Also the reason why a lipophilic membrane-permeable compound should have any membrane receptors at all remains unclear (126, 127), but this holds true for many

other lipids as well. Why does PA have two GPCRs? Is the function of GPCR41 different from that of GPCR43, and if not, then why are these two receptors expressed, in some cases, in the same cell type (e.g. adipocytes)? Finally, the affinity for PA is low and PA receptors need supraphysiological concentration of PA to mediate its effects. This raises the question whether GPCR41 and GPCR43 are the effective receptors for PA under normal physiological conditions. It is possible that PA as well as other SCFA act as surrogate agonists rather than endogenous agonists for GPCR41 and GPCR43, or it is possible, but less likely, that these receptors are activated in certain pathological situations when the PA concentration is unusually high, such as gingival inflammation and propionic acidemia. Disregarding these open questions, it is clear that PA is an important part of the communication between the microbiota and the physiology of the host and more recognition of this notion may prove useful for designing improved functional foods.

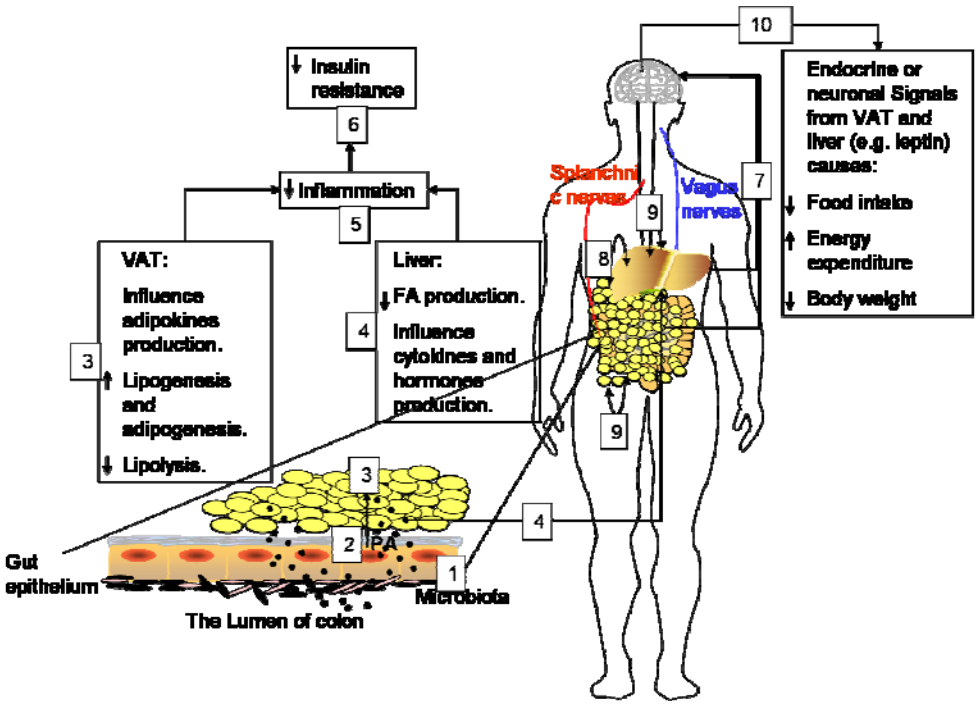

Figure 3: Schematic representation of a hypothetical model to explain the effects of PA. PA is produced by the fermentation of undigested food by the colonic microbiota (1). PA passes the gut epithelium (2) and reduces the production and the release of FA from (3) VAT and liver (4). This might lead to inhibition of inflammation (5) and consequently to insulin sensitivity (6). In addition, PA influences the production of hormones by VAT (3) and liver (4), which in turn initiates endo- (7), para- (8) and auto-crine (9) and neuronal pathways (via vagus and splanchnic nerves) to reduce food intake and to influence other metabolic effects (10).

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Chapter 3

Regulation of Adipokine Production in Human Adipose Tissue by Propionic Acid

Sa'ad H. Al-Lahham, Han Roelofsen, Marion Priebe, Desiree Weening, Martijn Dijkstra, Annemieke Hoek, Farhad Rezaee, Koen Venema and Roel J. Vonk

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Abstract

Background: Dietary fiber (DF) has been shown to be protective for the development of obesity, insulin resistance and type 2 diabetes. Short-chain fatty acids, produced by colonic fermentation of DF might mediate this beneficial effect. Adipose tissue plays a key role in the regulation of energy homeostasis, therefore, we investigated the influence of the short-chain fatty acid propionic acid (PA) on leptin, adiponectin and resistin production by human omental (OAT) and subcutaneous adipose tissue (SAT). As PA has been shown to be a ligand for G-protein coupled receptor (GPCR) 41 and 43, we investigated the role of GPCR's in PA signaling.

Materials and methods: Human OAT and SAT explants were obtained from gynecological patients who underwent surgery. Explants were incubated for 24 hours with PA. Adipokine secretion and mRNA expression were determined by ELISA and RT-PCR, respectively.

Results: We found that PA significantly stimulated leptin mRNA expression and secretion by OAT and SAT, whereas it had no effect on adiponectin. Furthermore, PA reduced resistin mRNA expression. Leptin induction, but not resistin reduction, was abolished by inhibition of Gi/o-coupled GPCR signaling. Moreover, GPCR41 and GPCR43 mRNA levels were considerably higher in SAT than in OAT.

Conclusions: We demonstrate that PA stimulates expression of the anorexigenic hormone leptin and reduces the pro-inflammatory factor resistin in human adipose tissue depots. This suggests that PA is involved in regulation of human energy metabolism and inflammation and in this way may influence the development of obesity and type 2 diabetes.

3.1 Introduction

Obesity and its associated disorders, such as insulin resistance, type 2 diabetes and cardiovascular diseases, have become major public health issues. The etiology of obesity and insulin resistance is complex and involves life style factors such as physical activity and diet. The dietary fiber content (DF) of the diet is one of the factors that may influence the development of these diseases. Several studies show that consumption of DF prevents accumulation of fat mass (1-3) and increases insulin sensitivity (4-6). This may be due to fermentation of DF by the colonic-microbiota leading to the production of metabolites such as short-chain fatty acids (SCFA). SCFA are rapidly absorbed from the colonic lumen and drains through the visceral tissues into the portal vein (7).

Recently it has been shown that SCFA, including PA, are ligands for the G protein-coupled receptor GPCR41 and GPCR43. These receptors, like other GPCR, are linked to GTP-binding proteins (G-proteins). G-proteins are attached to the cytoplasmic side of the receptor, where they serve as relay molecules functionally coupling the receptors to their downstream targets. G-proteins are classified into four major classes, namely Gs, Gi/o, Gq/11 and G12/13. Each of them is specific for a particular set of GPCR's and a particular set of downstream targets (8). Both GPCR41 and GPCR43 have been shown to be coupled to Gi/o-proteins and are present in adipose tissue (9;10), which suggests that adipose tissue is an important target for SCFA. As adipose tissue is an endocrine organ that produces a great variety of adipokines, that influence metabolism in other organs, it could constitute a link between colonic fermentation and peripheral metabolic effects.

The principal SCFA that are produced in the colon are acetic acid, propionic acid and butyric acid. However, most of the studies investigated the effects of butyric or acetic acids, while very little is known about effects of PA. In addition, 90% of butyric acid is utilized by colonocytes and very little reaches the

visceral tissues (11), while the majority of acetic acid and PA passes the colonocytes and visceral tissues and drains into the portal vein. Recently, it has been demonstrated that PA stimulates the production of the anorexigenic adipokine leptin in mouse and bovine adipocytes *in vitro* (12-14) and sheep adipose tissue *in vivo* (15). However, these and most of earlier studies were performed in animals or animal tissue, namely ruminants (16). Therefore, the aim of our study was to determine the effect of PA on the expression and secretion of adipokines by human adipose tissues. As different adipose tissue depots may vary in adipokine secretion patterns, we used omental as well as subcutaneous tissue. In addition to leptin, which is known to reduce food intake and increase energy expenditure (17) we examined the effect on adiponectin and resistin. Adiponectin has insulin sensitizing and anti-inflammatory effects (18). Resistin is a pro-inflammatory factor (19-21) and recently, it has been shown to be inversely associated with insulin sensitivity (22-24). PA is a ligand for GPCR41 and GPCR43. Consequently, we also investigated the involvement of the Gi/o-proteins signaling pathway in the regulation of adipokine response to PA treatment.

3.2 Materials and methods

3.2.1 Materials

Gentamycin, glucose, PA and pertusis toxin (PTX) were purchased from Sigma (Zwijndrecht, The Netherlands). M199 media was purchased from Invitrogen (Breda, The Netherlands). GPCR43 and GPCR41 primers were purchased from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands); whereas the other primers were purchased from Biolegio (Nijmegen, The Netherlands).

3.2.2 Human adipose tissue culture

Human omental and abdominal subcutaneous adipose tissue explants as well as serum samples were obtained from 28 females, who underwent surgery for gynecologic disorders such as myoma, endometriosis and refertilization. None of the women had diabetes and their characteristics are summarized in Table 1. The study was approved by the local medical ethical committee.

Table 1: Characteristics of adipose tissue donors (N=28).

Characteristics	Mean	Range
Age (years)	45.8 ± 9.3	26-68
body mass index (BMI, Kg/m^2)	26.3 ± 3.3	20.8-33.3
Waist circumference (WC, cm)	87.3 ± 11.4	57-104
Hip circumference (HC, cm)	102.4 ± 9.7	82-120
Waist to hip ratio (WHR)	0.9 ± 0.08	0.54-0.93

Adipose tissue culture was performed as described previously (25) with slight modifications. 0.5 g tissue/5 ml of M199 medium was used and after the last washing step, tissue explants were incubated for 24 hours with different PA concentrations (0, 0.01, 0.1, 1, 3 and 10 mM). Regarding PTX treatment, adipose tissue explants were pre-incubated with PTX (100 ng/ml) for 2 hours. Thereafter, 3 mM PA was added and incubated for 24 hours. Subsequently, tissue was frozen immediately in liquid nitrogen and then stored at -80°C until RNA was isolated. Media samples were stored at -80°C prior to ELISA measurements.

3.2.3 Relative Q-PCR analysis

Total RNA was isolated from adipose tissue by the RNeasy lipid tissue mini kit and cDNA was synthesized using the Quantitect kit (Qiagen, Venlo, Netherlands). Relative quantification of genes were performed in triplicate with the ABI 7900HT

sequence detection system for relative real time polymerase chain reaction (Taqman, Applied Biosystems) using the ΔΔCT method. The primers pairs and probes used are displayed in Table 2. Stability of several housekeeping genes was assessed by geNorm analysis software (http://medgen.ugent.be/~jvdesomp/genorm/) (26). GAPDH was chosen as the most stable housekeeping gene expressed in adipose tissue. PCR was performed using TaqMan Universal Master Mix in a total reaction mix volume of 10 µL. The PCR conditions were: 15 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C followed by 1 minute at 62 °C.

3.2.4 Adipokine protein quantification

Leptin and adiponectin concentrations were measured in culture media by Duoset ELISA kit in duplicate, according to the manufacturer's description (R&D Systems, Abingdon, United Kingdom); while resistin was measured in culture media and serum by Quantikine ELISA kit (R &D Systems).

3.2.5 Statistics

The average CV of measurements on triplicate incubations was 15.1% for mRNA and 28.1% for protein. All data are given as mean ± SEM. Comparison between two groups was performed by two-sided paired Student's t-test. The correlation between adipokine mRNA expression and protein level, age and anthropometric indices (body mass index (BMI), waist circumference (WC), hip circumference (HC) and waist to hip ratio (WHR), was calculated using the Pearson's product

moment correlation coefficient test. Results were considered to be statistically significant when $P < 0.05$.

Table 2: Primer sequences

Primer ID	Primer sequence (5'➔3')
Leptin forward	TCA CCA GGA TCA ATG ACA TTT CAC
Leptin reverse	AGC CCA GGA ATG AAG TCC AAA C
Leptin probe	CGC AGT CAG TCT CCT CCA AAC AGA AAG TCA
Adiponectin forward	AGG CCG TGA TGG CAG AGA T
Adiponectin reverse	GTC TCC CTT AGG ACC AAT AAG ACC T
Adiponectin probe	ATC TCC TTT CTC ACC CTT CTC ACC AGG G
Resistin forward	AAG CCA TCA ATG AGA GGA TCC A
Resistin reverse	CTC CAG GCC AAT GCT GCT TA
Resistin probe	CCC TAA ATA TTA GGG AGC CGG CGA CCT C
GAPDH forward	GGT GAA GGT CGG AGT CAA CG
GAPDH reverse	ACC ATG TAG TTG AGG TCA ATG AAG G
GAPDH probe	CGC CTG GTC ACC AGG GCT GC
GPCR41	Hs00271131_s1*
GPCR43	Hs00271142_s1*

*, ID numbers of primer sets from Applied Biosystems.

3.3 Results

3.3.1 PA dose-effect relationship with adipokine mRNA expression in omental adipose tissue

OAT explants derived from 13 subjects were used to determine the dose-effect relationship of PA on adipokine mRNA expression. OAT explants of each subject were incubated in triplicate with each concentration of PA for 24 hours. Figure 1 shows that leptin mRNA expression was significantly stimulated 65% and 100%

by 1 mM (P = 0.04) and 3 mM (P = 0.006) PA treatments, respectively. In contrast, 10 mM did not influence leptin mRNA expression. This was also noticed by Le Poul et al. (10), who found that 10 mM did not influence neutrophils chemotaxis, while 1 mM did. On the other hand, resistin mRNA expression was significantly down-regulated 44% (P = 0.0026) and 94% (P < 0.0001) in adipose tissue treated with 3 and 10 mM PA, respectively. None of the PA concentrations affected adiponectin mRNA expression.

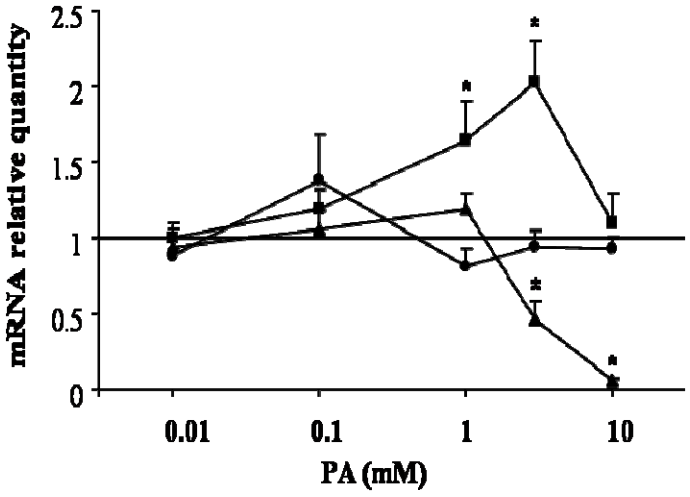

Figure 1: Dose-response curves of PA on adipokine production by human OAT *ex vivo*. OAT explants of each subject were incubated in triplicate with each concentration of PA for 24 hours. Leptin (■), adiponectin (●) and resistin (▲) mRNA expression levels were determined by RT-PCR and depicted as relative quantities compared to controls (without PA). Error bars represent SEM.* P < 0.05.

3.3.2 PA effect on adipokine mRNA expression and protein secretion by omental adipose tissue

Based on the results above, 3 mM PA was chosen for additional experiments in an extended set of explants to investigate the influence of age and anthropometric indices of the explant donors on the magnitude of the PA effect on adipokine mRNA expression. Moreover, mRNA response was validated on the protein level. As represented in Figure 2A, leptin mRNA (N=27) and protein (N=12) levels were significantly induced by ~90% (P < 0.0001 for both); whereas resistin expression

was significantly reduced (N=27; P < 0.0001) on the mRNA level by 46%, but not on the protein level (N=12; P = 0.61). With respect to adiponectin, neither mRNA (N=22) nor protein levels (N=12) were changed. Induction of leptin mRNA expression after PA treatment of OAT was independent of age or anthropometric indices of the donors (Table 3). Basal resistin mRNA expression in OAT was positively correlated to the donors' age (R = 0.49, *P* = 0.013) but not to anthropometric indices, whereas the response of resistin to PA was negative correlated to the donors' age only (R = -0.41, *P* = 0.03). While there was a positive correlation between basal resistin mRNA expression in adipose tissue with age, no correlation was observed between serum resistin and age of the explant donors.

Table 3: Analysis of the correlations (r) in OAT between the mRNA expression levels of GPCR41, GPCR43 and resistin, resistin level in serum and resistin and leptin responses to 3 mM PA on the one hand and age and anthropometric indices on the other hand.

	GPCR41 basal mRNA levels	**GPCR43 basal mRNA levels**	**Resistin basal mRNA levels**	**Resistin basal serum levels**	**Resistin mRNA response to PA**	**Leptin mRNA response to PA**
BMI	0.078	-0.23	0.088	0.29	-0.3	0.037
WC	0.099	-0.37	0.076	0.17	-0.14	0.11
HC	-0.15	-0.53*	0.22	0.17	-0.17	-0.17
WHR	0.48*	0.2	-0.083	0.035	-0.025	0.32
Age	-0.023	0.05	0.49*	0.25	-0.41*	-0.015

BMI: Body mass index, HC: hip circumference, WC: waist circumference and WHR: waist-hip ratio. * $P < 0.05$.

3.3.3 PA effect on adipokine mRNA expression and protein secretion by subcutaneous adipose tissue

The effect of PA was also investigated on adipokine mRNA and protein levels in SAT derived from 5 and 12 subjects, respectively. As depicted in Figure 2B, leptin protein and mRNA levels were induced by approximately 35% (*P* = 0.012 and 0.027, respectively), resistin was reduced only on the mRNA level by 32% (*P* = 0.003) and adiponectin was unaffected both on the mRNA and protein level.

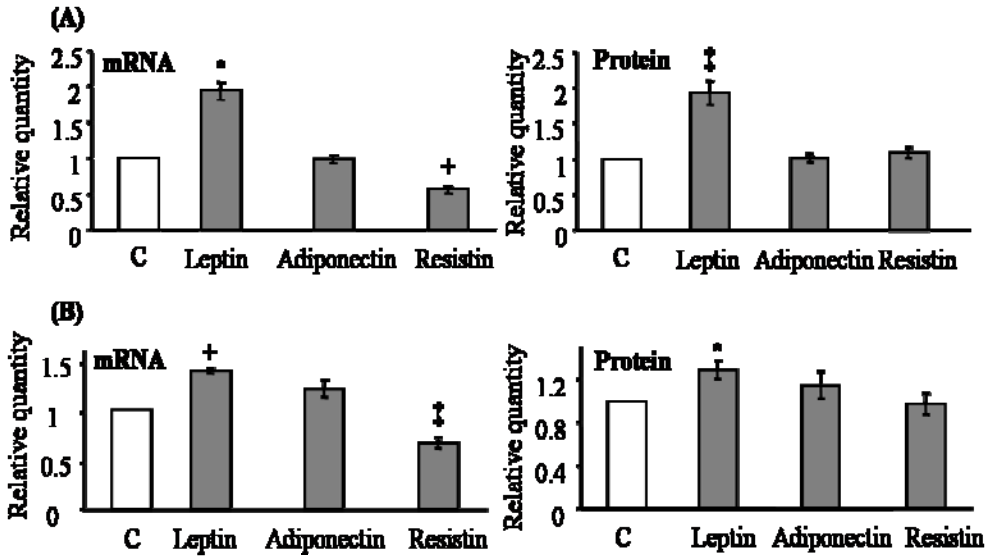

Figure 2: PA effects on adipokine production by OAT and SAT *ex vivo*. 3 mM PA (■) was chosen to validate the effect of PA on adipokine production by OAT (A) and SAT (B) on both mRNA and protein levels. OAT and SAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. mRNA expression levels were determined by RT-PCR and secreted adipokine levels in the media were determined by ELISA. Results are depicted as relative quantities compared to controls (without PA; □). Error bars represent SEM. ‡ $P < 0.05$; * $P < 0.001$ and + $P < 0.0001$ and ‡ $P < 0.05$.

GPCR41 and GPCR43 expression in human omental and subcutaneous adipose tissues

SCFA are ligands for the G protein-coupled receptor GPCR41 and GPCR43 (9;10). Therefore, we determined their expression in human OAT and SAT. As shown in Figure 3, we found that GPCR41 was expressed at similar levels as GPCR43 in each adipose depot. However, levels of both receptors in SAT were approximately 10-fold higher than in OAT (P = 0.009 and 0.021 for GPCR41 and GPCR43, respectively). We also determined whether GPCR41 and GPCR43 expression in OAT (N=21, 3 biopsies/subject) was correlated to age and anthropometric indices of the donors (Table 3). GPCR41 mRNA expression had a significant and positive correlation with WHR (R = 0.48, P = 0.03); whereas GPCR43 expression correlated negatively to HC (R = -0.53, P = 0.013).

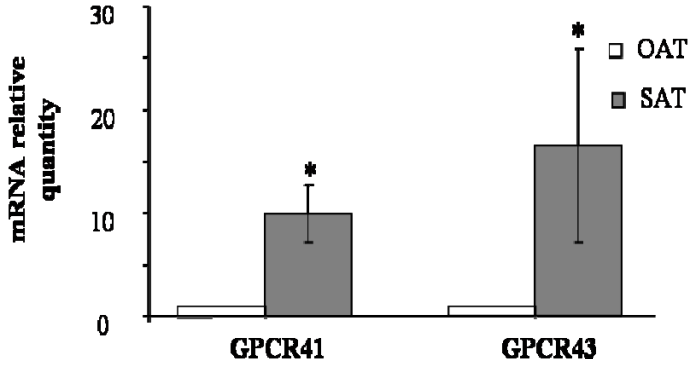

Figure 3: Gene expression levels of GPCR41 and GPCR43 in OAT and SAT *ex vivo*. mRNA expression of each receptor in SAT was determined by RT-PCR and depicted as a relative quantity compared to OAT. Error bars represent SEM. * $P < 0.05$.

3.3.4 Involvement of Gi/o-protein coupled receptors in the effect of PA on leptin and resistin mRNA expression

In mouse adipocytes, it has been shown that PA mediates its effect on leptin production via Gi/o-protein coupled receptor (Gi/o-PCR) (12). To determine whether PA uses GPCR coupled to Gi/o proteins to affect leptin and resistin expression in human adipose tissue, OAT and SAT biopsies taken from six and five subjects, respectively, were pretreated with PTX for 2 hours to block the Gi/o pathway and then they were treated with or without 3 mM PA for 24 hours. Figure 4 illustrates that induction of leptin expression by PA was completely abolished by PTX pretreatment in both OAT and SAT, while the PA-induced reduction in resistin expression was not affected by PTX. PTX treatment also reduced baseline leptin expression by approximately 60% and 40% in both OAT and SAT, respectively (P = 0.004 and 0.01, respectively). In OAT, PA plus PTX could not completely abolish leptin mRNA expression to the level observed with PTX alone.

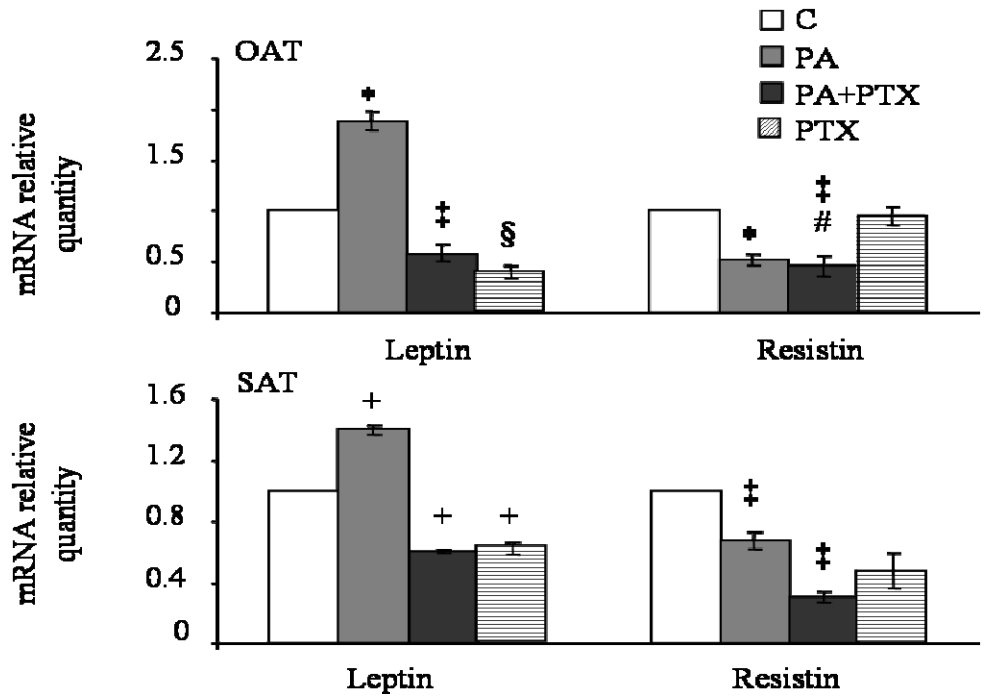

Figure 4: Involvement of G-protein coupled receptor(s). The involvement of G-protein coupled receptors in mediating PA effects on adipokines expression was determined by blocking the Gi/o signaling pathway with PTX. OAT and SAT explants of each subject were incubated in triplicate with PTX (100 ng/ml) for 2 hours before incubation for 24 hours with or without 3 mM PA. mRNA expression levels were determined by RT-PCR and depicted as relative quantities compared to controls (without PA). Error bars represent SEM. * $P \leq 0.001$ vs control; ‡ $P < 0.01$ vs control; § $P < 0.01$ vs PA + PTX, # $P < 0.05$ vs PTX and + $P < 0.05$ vs control.

3.4 Discussion

The present human *ex vivo* study reveals that PA influences adipokine production by both adipose tissue depots (OAT and SAT). Explants were derived from women without diabetes with BMI's ranging between 20.8 and 33.3 Kg/m^2. PA induced leptin, both on the mRNA and protein level to a similar extent, while it almost completely abolished resistin mRNA expression. On the other hand, adiponectin

expression was not influenced. The magnitude of the PA effect on leptin was 2.6 fold higher in OAT compared to SAT and was independent of the degree of adiposity and age of the explant donors. Induction of leptin by PA may indicate that PA reduces food intake and increases energy expenditure, since leptin is a potent anorexigenic hormone (17). In sheep, PA has been shown to induce leptin expression in adipose tissue (15) and to reduce food intake (16;27). The observed PA-induced reduction of resistin mRNA expression was slightly more pronounced in OAT compared to SAT and was negatively associated with age but independent of the degree of adiposity of the explant donors. However, on the protein level no significant change in resistin expression was seen. This discrepancy between the mRNA and the protein level may be explained by the fact that resistin is only released from the cell surface membrane or intracellular stores after an inflammatory trigger (27). Therefore, a reduction in resistin protein only can be observed when cellular stores are depleted. Resistin is pro-inflammatory and, in human adipose tissue, is predominantly produced by macrophages (28). Resistin is also a marker for insulin resistance in humans (21-23). The PA-induced decrease in resistin expression may imply that PA is acting as an anti-inflammatory agent. This is in agreement with other studies in which PA has been shown to have anti-inflammatory effects on human neutrophils and mouse colonic organ cultures (29). Furthermore, PA inhibits proliferation of granulocyte/macrophage, hematopoietic progenitor cells and lymphocyte activation (30-33). The observed improvement of insulin sensitivity in humans after high DF diets (4-6) may be attributed to this anti-inflammatory effect of PA since in obese persons adipose tissue inflammation and insulin resistance are closely linked (34). In addition to PA, we investigated other SCFA. Our preliminary results show that other SCFA may also have an effect on adipokine expression (not shown). This is the focus of our future studies.

The expression of GPCR41 and GPCR43 by human adipose tissue was shown to be controversial. For example, Le Poul et al (10) showed that GPCR41 and GPCR43 were expressed in human adipose tissue, while Hong et al (13)

showed it was not expressed. In addition, in these previous studies the adipose tissue depot was not specified. Our results show that both human adipose tissue depots expressed GPCR41 and GPCR43 mRNA. SAT expressed approximately 10-fold higher amounts of each receptor than OAT, suggesting that SAT is more responsive to PA than OAT. However, we found slightly higher responses to PA in OAT compared to SAT. Furthermore, we found that GPCR41 mRNA level in OAT was positively associated with WHR, as a measure of adiposity, while GPCR43 was negatively associated with HC (Table 3). However, no correlation with other measures of obesity (BMI and WC) was found. Therefore, the relevance of this observation remains unclear.

PTX abolished leptin induction by PA in both human OAT and SAT similar to what has been observed in mouse adipocytes (12). This suggests that PA regulates leptin production through GPCR coupled to the Gi/o signaling pathway. However, the effect of PA on resistin expression was not inhibited by PTX. Le Poul et al. (10) showed that there is a unique Gi/o coupling for GPCR41 but for GPCR43, a dual coupling exists through Gi/o and Gq. Furthermore, in human adipose tissue, resistin is almost exclusively expressed in macrophages (35) while leptin expression is specific for adipocytes (36). This may suggest that leptin induction by PA is mediated via GPCR41 on adipocytes, coupled to Gi/o, while the reduction of resistin by PA is mediated via GPCR43 on resident macrophages, coupled to Gq.

In *in vitro* studies (30-33), including our study, relatively high PA concentrations (1-10 mM) are needed for the observed effects. The question rises whether colonic fermentation can provide such concentrations. Due to the absence of blood flow, the effective concentration in tissue cultures probably is substantially higher than *in vivo.* The PA concentration in the human colon is approximately 20 mmol/kg (7). In ruminants, 50% of absorbed PA reaches the portal vein (37), indicating that the remainder is utilized by tissues it encounters

such as the colonic wall and possibly OAT. I*n vivo,* the exposure time of adipose tissue to PA may be an important factor. Furthermore, effects of different SCFA may be additive as acetate, propionate and butyrate all share the same receptors (9;10). Further *in vivo* measurements have to confirm this.

In conclusion, the present study demonstrates for the first time that PA influences adipokine secretion by human adipose tissue. We show that leptin and resistin, but not adiponectin production is affected by PA and involves GPCR signaling. These findings suggest that modulation of PA quantity through e.g. dietary manipulation, prebiotics and probiotics, or dietary supplementation may have potential in preventing obesity and its associated complications such as inflammation and insulin resistance.

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Chapter 4

Propionic Acid Links Microbiota to Human Adipose Tissue Inflammation

Sa'ad Al-Lahham, Han Roelofsen, Farhad Rezaee, Desiree Weening, Annemieke Hoek, Roel Von and Koen Venema

In preparation

Abstract

Adipose tissue is a primary site of obesity-induced inflammation which is emerging as an important contributor to obesity-related diseases. The factors influencing adipose tissue-induced inflammation and the resulting pathologies remain poorly understood. However, dietary fiber diets appear to be protective, suggesting a cross-talk between the colonic metabolism and adipose tissue-induced inflammation. Short-chain fatty acids, e.g. propionic acid (PA), are the principal products of the dietary fiber fermentation by microbiota. Here we show that the treatment of human omental adipose tissue with PA results in a significant downregulation of several inflammatory parameters and positively influences factors associated with insulin sensitivity, adipogenesis and lipogenesis in this tissue via Gi/o-dependent and –independent mechanisms. The negative cross-talk between microbiota-produced PA and the adipose tissue inflammatory process provides a new paradigm in understanding the relation between the microbiota and adipose tissue physiology and suggests its potential in preventing obesity-related inflammation and -diseases.

4.1 Introduction

Obesity is associated with chronic activation of low-grade inflammation (1), which is implicated in the pathogenesis of obesity-associated diseases, including insulin resistance, type-2 diabetes (2), cardiovascular diseases (3;4). The etiology of obesity and low grade inflammation is complex and involves biological and environmental factors. Recently, it has been shown that specific members of the microbiota (specifically *Firmicutes*) in humans are associated with obesity and associated pathology (5-7). Furthermore, the colonization of germ-free mice with microbiota from obese mice results in significantly greater adiposity than colonization with microbiota from lean mice (8). Conversely, so-called prebiotic diets in general and long chain O-linked oligofructoses (fructans) in particular (9) are associated with general better health, including the decrease in body weight, fat mass and the severity of diabetes (10-12). The mechanisms by which the intestinal microbiota composition and metabolism can have such large consequences on obesity-related pathophysiology remain, however, obscure at best.

Fermentation of dietary fiber/ resistant starch by the colonic microbiota is the primary source for the production of short-chain fatty acids (SCFA), in particular acetic acid, butyric acid, and propionic acid. It has been demonstrated that SCFA influence the physiology of humans, such as inhibition of inflammation, protection from cancer, promotion of satiety and fuels for cells growth (13-15). However, most of the studies focused on butyrate and to a lesser extent on acetate, while the effects of propionic acid (PA) in humans have remained largely unexplored. In this study we focus on PA; the majority of the PA quantity produced in the colon (20 mmol/kg) (16) is absorbed, by-passes the colonocytes and the viscera, and drains into the portal vein. The PA concentration in the portal vein of non-fasting humans was demonstrated to be approximately 0.1 mM (16-18); however, the quantity that is absorbed or interacts with visceral tissues, e.g.

omental adipose tissue (OAT), has not been examined yet but is expected to reach the millimolar level. Together, it is fair to say that PA is a potential microbiota-derived metabolite to interact with the physiology of the host tissues, such as OAT.

Indeed, important evidence for PA to act as an effecter on body-wide physiology exists. In 2003, three independent studies found that PA and other SCFA are potent and efficacious ligands for the until then orphan G-protein coupled receptors 41 (GPCR41) and 43 (GPCR43) (19-21). GPCR41 and GPCR43, like other GPCRs, are linked to GTP-binding proteins (G-proteins). G-proteins serve as relay molecules functionally coupling the receptors to their downstream targets and include four major classes, namely Gs, Gi/o, Gq/11 and G12/13. Very recently Maslowski et al (22) generated GPCR43 knockout mice and established the importance of GPCR43 as an anti-inflammatory receptor. GPCR43 knockout mice showed exacerbated inflammation in models of inflammatory diseases, i.e. colitis, arthritis and asthma. Wild type germ-free mice, which are devoid of bacteria and therefore contain little or no SCFA showed a similar dysregulation of certain inflammatory responses (16). These experiments strongly support the role of PA production by the microbiota as an important anti-inflammatory mechanism.

As adipose tissue is a major contributor to obesity induced low-grade inflammation (23-25) and as both GPCR receptors of PA are present on adipose tissue one could envisage that PA could constitute the elusive link between colonic-microbiota metabolism and low-grade adipose tissue-induced inflammation in humans, prompting direct investigation of this hypothesis. In the present study we investigated the influence of PA on several inflammatory parameters in human OAT explants. It appeared that physiologically relevant concentrations of PA reduced the production in OAT of a panel of chemokines, as well as cytokines and adipose tissue macrophage markers. Most of these effects became less pronounced when OAT tissue from obese patients was tested for sensitivity to PA. Functionally, PA treatment improved insulin sensitivity, adipogenesis and

lipogenesis in adipose tissue. At a mechanistic level, we identified Gi/o-dependent PA-induced signal transduction as the responsible pathway for at least part of these effects. Thus, we provide evidence that a microbial metabolite, *in casu* PA, can counteract the inflammatory reaction in human adipose tissue, providing for the first time evidence that microbiological metabolites can influence the physiology of the human adipose compartment and suggesting that PA may have potential in preventing obesity-related inflammation and diseases.

4.2 Materials and Methods

4.2.1 Materials

Gentamycin, glucose, PA and pertusis toxin (PTX) were purchased from Sigma (Zwijndrecht, The Netherlands). M199 media was purchased from Invitrogen (Breda, The Netherlands). Preadipocytes and their media were purchased from PromoCell (Uithoorn, The Netherlands). CD16A, CD31, CD163, and MMP-9 primers were purchased from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands); whereas the rest of the primers were purchased from Biolegio (Nijmegen, The Netherlands).

4.2.2 Human adipose tissue and cell culture

OAT explants were obtained from women who underwent surgery for gynecologic disorders such as myoma and prolapse. None of the women had diabetes and their anthropometric indices are illustrated in Table 1. The study was approved by the local medical ethical committee. Adipose tissue culture was performed as described previously (36) with slight modifications. After the last washing step, tissue explants were incubated for 24 hours with or without 3 mM PA. Regarding

PTX treatment, adipose tissue explants were pre-incubated with PTX (100 ng/ml) for 2 hours; thereafter, 3 mM PA was added and incubated for 24 hours. Subsequently, tissue was snap frozen in liquid nitrogen and then stored at -80°C until RNA was isolated. Media samples were stored at -80°C prior to ELISA measurements. With respect to human preadipocytes, they were cultured and differentiated into adipocytes according to PromoCell instructions.

Table 1: Anthropometric indices of adipose tissue donors.

Subject ID	BMI	WHR	WC
S-1	30,44	0,82	95
S-2	27,46	0,86	90
S-3	30,85	0,93	95
S-4	31,89	0,91	104
S-5	23,59	0,84	82

4.2.3 Relative Q-PCR analysis

Total RNA was isolated by the RNeasy lipid tissue mini kit and cDNA was synthesized using the Quantitect kit (both from Qiagen, Venlo, Netherlands). Relative quantification of genes were performed in triplicate with the ABI 7900HT sequence detection system for relative real time polymerase chain reaction (Taqman, Applied Biosystems) using the ΔΔCT method. The primers pairs and probes used are displayed in Table 2. Stability of several housekeeping genes was assessed by geNorm analysis software (http://medgen.ugent.be/~jvdesomp/genorm/) (37). GAPDH was chosen as the most stable housekeeping gene expressed in adipose tissue. PCR was performed using TaqMan Universal Master Mix in a total reaction mix volume of 10 µL. The PCR conditions were: 15 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C followed by 1 minute at 62 °C.

Table 2: The sequences of the primers.

Primer ID	Primer sequence (5'(3')
GAPDH forward	GGT GAA GGT CGG AGT CAA CG
GAPDH backward	ACC ATG TAG TTG AGG TCA ATG AAG G
GAPDH probe	CGC CTG GTC ACC AGG GCT GC
IRS-1 forward	CCA CTC GGA AAA CTT CTT CTT CAT
IRS-1 backward	GAG TCA TCC ACC TGC ATC CA
IRS-1 probe	AGG TGG GCC GTT CTG CCG TG
GLUT4 forward	GCT GTG GCT GGT TTC TCC AA
GLUT4 backward	CCC ATA GCC TCC GCA ACA TA
GLUT4 probe	CGA GCA ACT TCA TCA TTG GCA TGG GTT
FASN forward	GCA AAT TCG ACC TTT CTC AGA AC
FASN backward	GGA CCC CGT GGA ATG TCA
FASN probe	CCC GCT CGG CAT GGC TAT C
LPL forward	TGG AGA TGT GGA CCA GCT AGT G
LPL backward	CAG AGA GTC GAT GAA GAG ATG AAT G
LPL probe	CTC CCA CGA GCG CT
SREBP1c forward	GGA TTG CAC TTT CGA AGA CAT G
SREBP1c backward	AGC ATA GGG TGG GTC AAA TAG G
SREBP1c probe	CAG CTT ATC AAC AAC CAA GAC AGT GAC TTC CC
Pref-1 forward	TCC TGC GCG TCC TCT TG
Pref-1 backward	GAA GCA TTC AGC CCC ATA GG
Pref-1 probe	CTG CTG GCT TTC GGC CAC AGC
CD163 forward	TGC AGA AAA CCC CAC AAA AAG
CD163 backward	CAA GGA TCC CGA CTG CAA TAA
CD163 probe	AAC AGG TCG CTC ATG CCG TCA GTC A
CD16	Hs01569121_m1*
CD31	Hs01065282_m1*
MMP-9	Hs00234579_m1*

*, ID numbers of primer sets from Applied Biosystems.

4.2.4 Protein quantification

Secreted chemokines and cytokines were measured in culture media by multiplex-ELISA assay according to the manufacturer's description (Bio-Rad, Hercules, CA, USA).

4.2.5 Statistics

Comparison between two groups was performed by two-sided paired Student's t-test. Results were considered to be statistically significant at $P < 0.05$. The correlation between the production level of the examined parameters and anthropometric indices (i.e. body mass index (BMI), waist circumference (WC) and waist to hip ratio (WHR)) was calculated using the Pearson's product moment correlation coefficient test.

4.3 Results

4.3.1 PA counteracts inflammation in human adipose tissue

Adipose tissue is an essential endocrine organ that produces various adipokines, which are involved in the regulation of various physiological functions. During obesity, the production of several inflammatory parameter (23-25) and the infiltration of inflammatory cells is increased in adipose tissue, providing clear evidence that adipose tissue-induced inflammation is a principal factor in the pathogenesis of obesity related diseases. Concomitantly, it is becoming clear that especially prebiotic diets, favoring the production of SCFA by the colonic microbiota, are associated with reduced obesity related diseases (26;27). Hence, we investigated the influence of 3 mM PA, a microbiota derived metabolite, on the production of several inflammatory parameters in human explants derived from 5 women, in triplicate. We found that PA treatment significantly ($P < 0.05$) downregulated the secretion of interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), granulocyte-colony stimulating factor (G-CSF), interferon-gamma-

induced protein (IP-10), Macrophage Inflammatory Proteins-1α and -1β (MIP-1α and MIP-1β) and "regulated upon activation, normal T cell expressed and secreted" (RANTES) approximately by 2 fold (Figure 1), while IL-1β and IL-4 were not influenced and IL-12 and IL-13 were not detected. It appeared that especially the inhibition of IL-10 and TNF-α was positively associated with the indicators of obesity, while the inhibition of G-CSF, MIP-1α, MIP-1β and RANTES was negatively associated and the response of IP-10 was not associated with any indicators (Figure 2). Moreover, the secretion of IL-10, MIP-1α, MIP-1β, RANTES and TNF-α from unstimulated explants were associated positively with some of the indicators of obesity, while G-CSF and IP-10 were negatively associated (Figure 3).

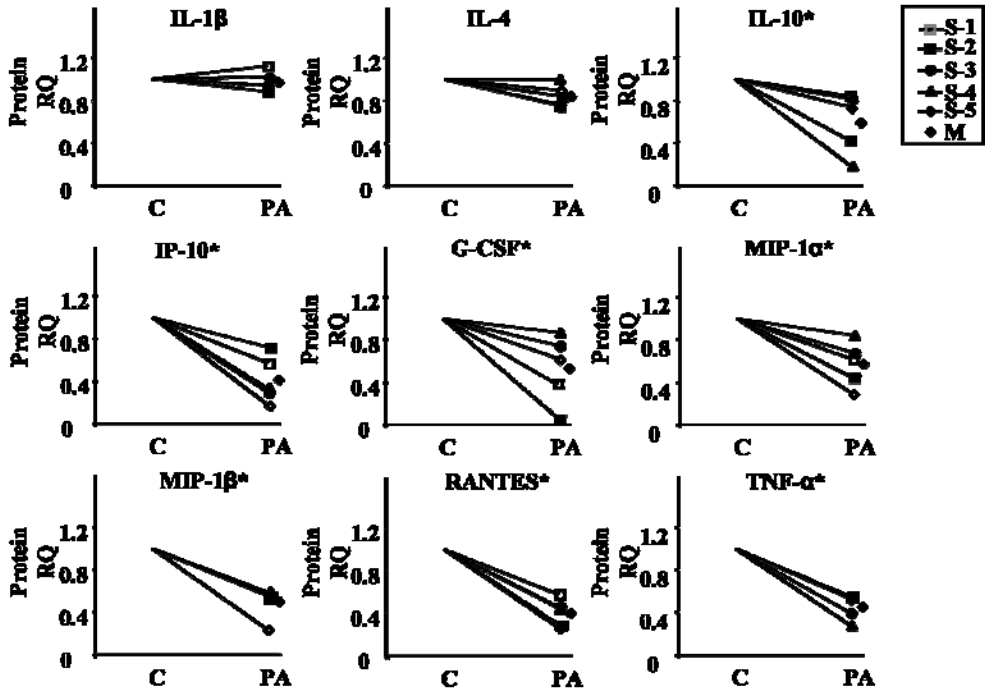

Figure 1: The influence of PA on the secretion of chemokines and cytokines by omental human adipose tissue. OAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. PA inhibited the secretion of IL10, IP-10, G-CSF, MIP-1α, MIP-1β, RANTES and TNF-α, while it had no effect on IL-1β and IL-4. Secreted quantities of chemokines and cytokine in the media were determined by multiplex-ELISA. Results are depicted as relative quantities (RQ) compared to the control (without PA; C). *, $P < 0.05$. M, mean.

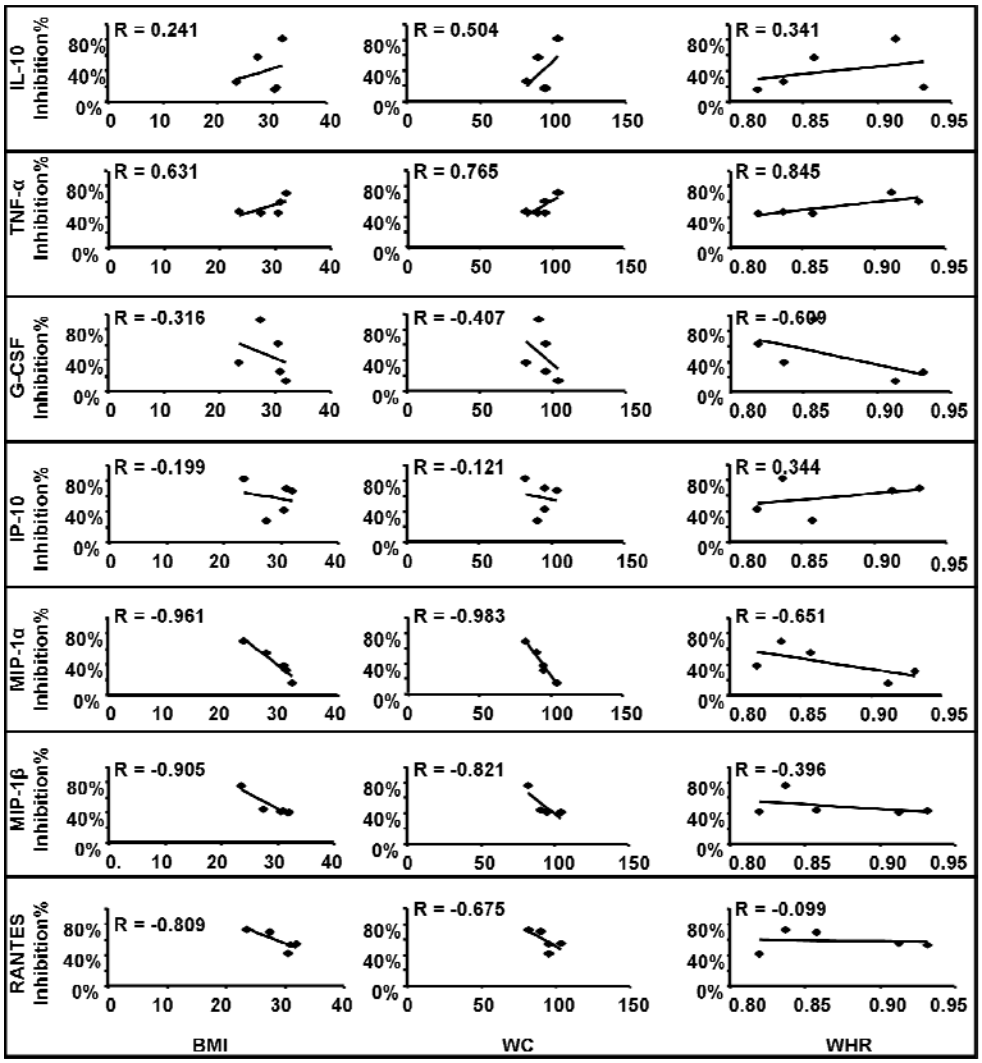

Figure 2: The association between the indicators of obesity and the response of chemokines and cytokines to PA treatment. OAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. The correlation coefficients (R) were determined between percentage of inhibition and the indicators of obesity, i.e. BMI, WC and WHR. The inhibition of IL-10 and TNF-α was positively associated with at least 2 indicators of obesity, while the inhibition of G-CSF, MIP-1α, MIP-1β and RANTES was negatively associated and the response of IP-10 was not associated with any indicators. Secreted quantities of chemokines and cytokine in the media were determined by multiplex-ELISA. Results are depicted as a percentage of inhibition compared to the control (without PA; C), which was set to a 100%.

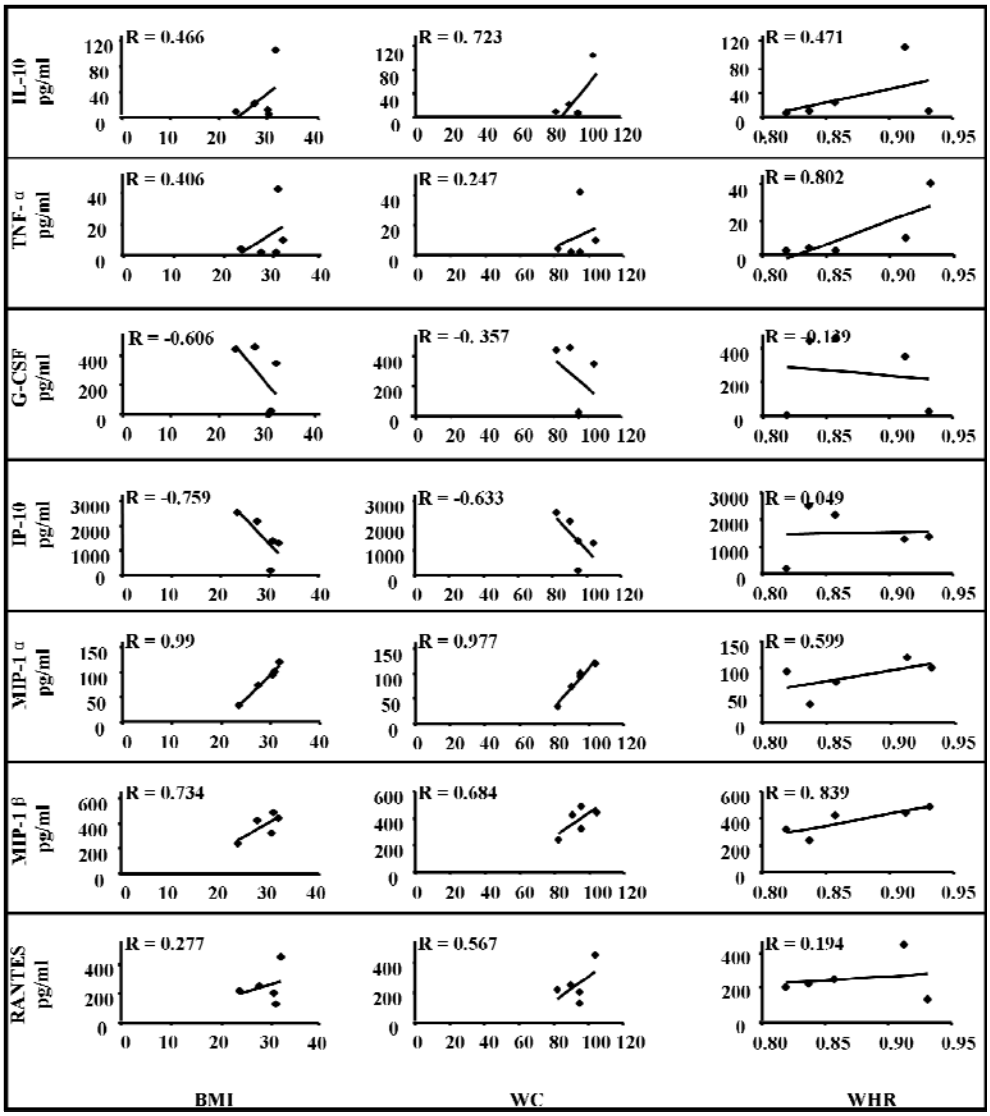

Figure 3: The association between the indicators of obesity and the basal quantity of chemokines and cytokines. Secreted quantities of chemokines and cytokines in the media from unstimulated OAT explants (in triplicate) derived from 5 subjects were determined by multiplex-ELISA and correlated to the indicators of, i.e. BMI, WC and WHR. The secretion of IL-10, MIP-1α, MIP-1β, RANTES and TNF-α was associated positively with at least one of these indicators, while G-CSF and IP-10 were negatively associated.

The observed inhibitory effect on the examined chemokines suggests that PA inhibits the recruitment, growth and/or activity of macrophages and/or transdifferentiation of preadipocytes into macrophages. To further clarify this, we

determined the effects of PA on the mRNA expression of adipose tissue macrophages (ATM) markers, CD16, CD31, CD163 and metalloproteinase 9 (MMP-9) (28-31) by human adipose tissue explants obtained from 4 women. 3 explants per subject were incubated with or without PA. As illustrated in Figure 4, the treatment significantly downregulated the mRNA expression of CD16, CD31, CD163 and MMP-9, by approximately 30%, 15%, 50%, and 59%, respectively ($P < 0.05$).

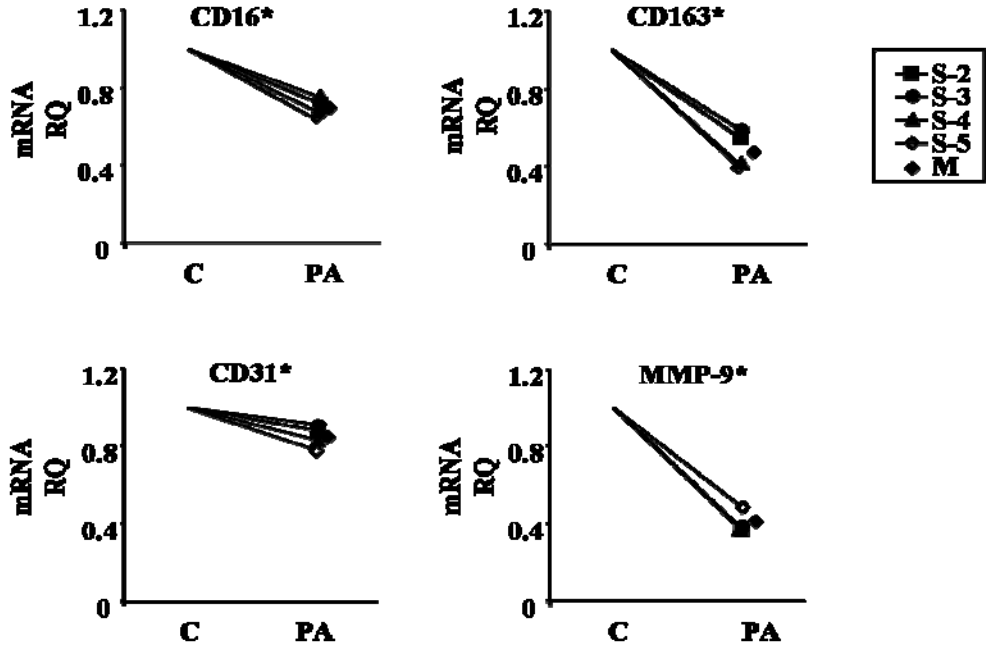

Figure 4: The influence of PA on the mRNA expression of adipose tissue macrophage related markers. OAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. PA downregulated all of the ATM markers, i.e. CD16, CD31, CD163 and MMP-9. The mRNA expression levels were determined by RT-PCR and depicted as relative quantities (RQ) compared to the control (without PA; C). *, $P < 0.05$. M, mean.

The inhibition of CD16, CD31 and CD163 was negatively associated with most of the indicators of obesity, while the inhibition of MMP-9 was positively associated (Figure 5). The basal levels of CD16, CD31 and MMP-9 in the unstimulated human adipose tissue explants were positively associated with at least one of the

indicators of obesity, while the level of CD163 was negatively associated with all of the indicators (Figure 6). To determine the contribution of adipocytes we investigated whether they express these markers. These adipocytes were obtained via the differentiation of omental preadipocytes. As depicted in Table 3, the mRNA expression of CD16 was not detected in adipocytes; while it was expressed by adipose tissue (CT = 32.2). CD31, CD163 and MMP-9 mRNA molecules were expressed in adipose tissue 1859-, 628-, and 2418-fold higher, respectively than in adipocytes (Table 1; $P < 0.05$) providing evidence that, at least partially, ATM contribute to the observed anti-inflammatory effects of PA, but not adipocytes.

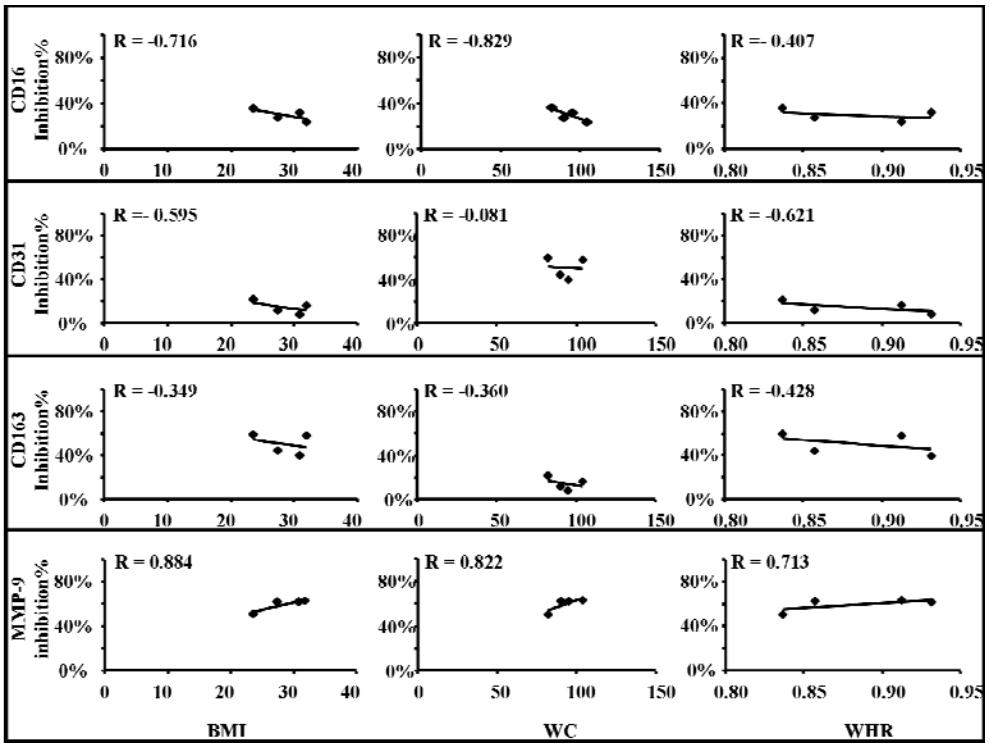

Figure 5: The association between the indicators of obesity and the response of adipose tissue macrophage related markers to PA treatment. OAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. Results are depicted as a percentage of inhibition compared to the control (without PA; C), which was set to a 100%. The correlation coefficients (R) were determined between percentage of inhibition and the indicators of obesity, i.e. BMI, WC and WHR. The inhibition of CD16, CD31 and CD163 was negatively associated with these indicators, while the inhibition of MMP-9 was strongly positively associated.

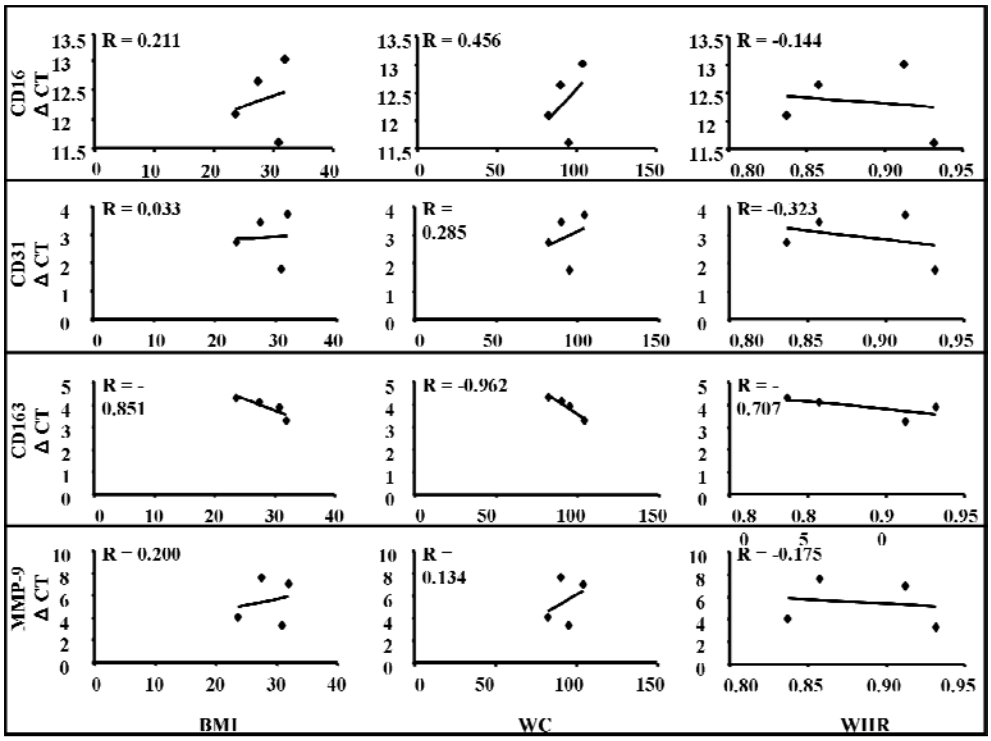

Figure 6: The association between the indicators of obesity and the basal mRNA level of adipose tissue macrophage related markers. OAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. The mRNA expression levels were determined by RT-PCR and depicted as ΔCT, which is calculated by subtracting the cycle threshold (CT) of each gene from the CT of the house keeping gene (GAPDH). The correlation coefficients (R) were determined between the values of ΔCT and the indicators of obesity, i.e. BMI, WC and WHR. The basal level of CD16, CD31 and MMP-9 were positively associated with at least one of these indicators, while CD163 was strongly negatively associated with all of indicators.

Table 3: Comparing the expression of adipose tissue macrophage markers between human primary adipocytes (HPA) and adipose tissue

Gene ID	HPA-CT value	OAT-CT value *	RQ
CD16A	ND	31.7	
CD31	33.2	22.3	1858.8
CD163	32.6	23.3	628.4
MMP-9	34.8	23.6	2417.7

CT, cycle threshold. *$P < 0.05$ vs HPA for all mentioned genes.

4.3.2 Propionic acid positively influences factors associated with insulin sensitivity in human adipose tissue explants

The anti-inflammatory influence of PA on the adipose tissue may provide a mechanistic explanation as to the preventive influence of diets leading to the microbial production of SCFA on the development of metabolic syndrome (32;33). If this notion is true, however, PA should negatively influence the associated-markers of metabolic syndrome. Therefore we decided to study the effects of PA on markers associated with adipogenesis, lipogenesis and insulin sensitivity in OAT explants. 3 explants per subject (taken from 4 women) were incubated with or without PA. It appeared that these explants all reacted to such treatment by significant upregulation of lipoprotein lipase (LPL; a lipogenesis marker) and sterol regulatory-element-binding protein-1c (SREBP-1c; another lipogenesis marker) mRNA expression by approximately 50% and 70%, respectively, while preadipocyte factor (Pref-1, an anti-adipogenesis marker) was significantly reduced by approximately 32% ($P < 0.05$) (Figure 7). GLUT-4, a factor associated with insulin sensitivity, was significantly induced by 75% (Figure 7).

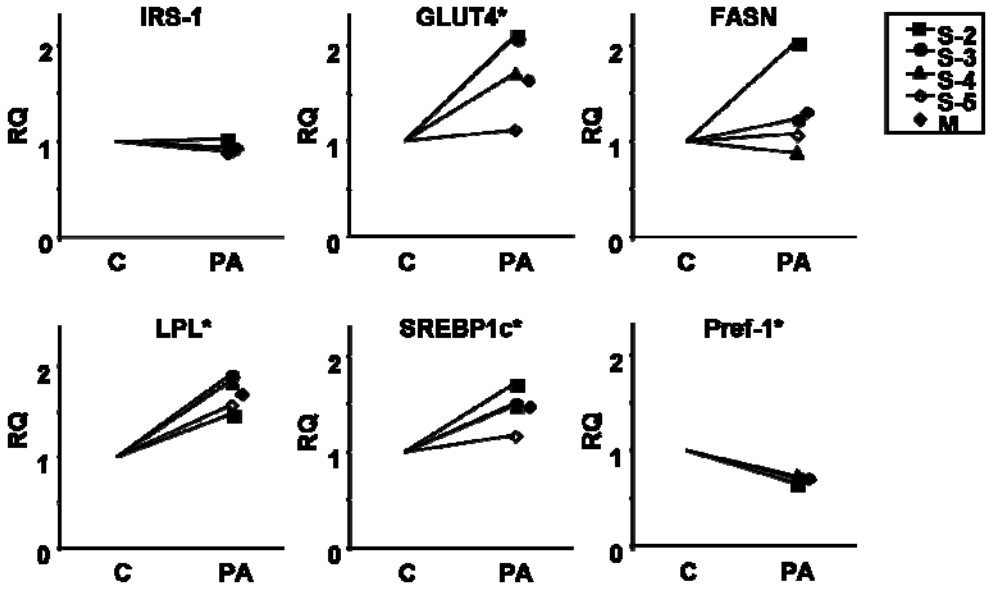

Figure 7: The influence of PA on the mRNA expression of lipogenesis, adipogenesis and insulin sensitivity related markers. OAT explants of each subject were incubated in triplicate with or without 3 mM PA for 24 hours. PA upregulated the expression of LPL, SREBP-1c and GLUT-4, while Pref-1 was reduced. The mRNA expression levels were determined by RT-PCR and depicted as relative quantities (RQ) compared to the control (without PA; C). *, $P < 0.05$. M, mean.

4.3.3 Propionic acid acts on the adipose tissue partially through Gi/o-dependent signal transduction

PA is a ligand for both GPCR41 and GPCR43, which both activate Gi/o-proteins to mediate PA signaling (19-21). As a consequence, we examined the role of the Gi/o-signal transduction pathway in the response of all examined factors in OAT toward PA treatment. As depicted in Figure 8, the treatment of OAT explants with the specific Gi/o inhibitor, pertussis toxin (PTX), abolished the response of IL-10, MIP-1α, MIP-1β, RANTES, G-CSF, TNF-α, Pref-1 and SREBP1c. On the other hand, PTX-pretreatment did not abolish the response of adipose tissue with respect to IP-10, CD16, CD31, CD163, MMP-9, LPL and GLUT-4. As PTX sensitivity is

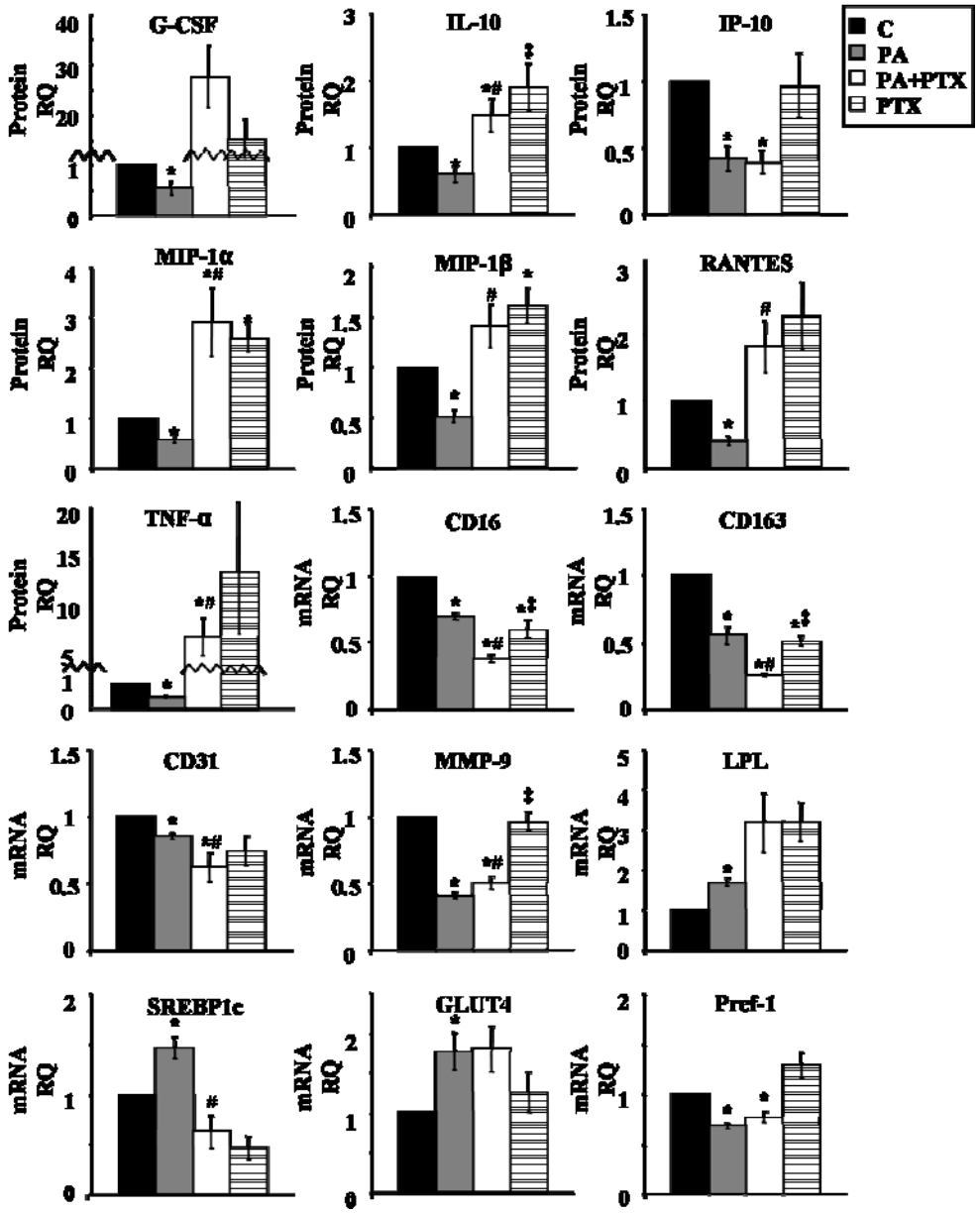

Figure 8: The involvement of Gi/o-signal transduction. OAT explants of each subject were incubated in triplicate with an inhibitor of Gi/o-protein (PTX) for 2 hours to block the Gi/o-signal transduction, and then OAT explants were treated with or without 3 mM PA for 24 hours. PTX abolished the response of IL-10, MIP-1α, MIP-1β, RANTES, G-CSF, TNF-α, Pref-1 and SREBP1c, while the others were not influenced. The mRNA expression levels were determined by RT-PCR and depicted as relative quantities (RQ) compared to controls (without PA; C). Data are represented as mean ± SEM. *, $P < 0.05$ vs C; #, $P < 0.05$ vs PA; ‡, $P < 0.05$ vs PA+PTX.

the defining hall mark of Gi/o-dependent signal transduction, these results show that the cross talk between PA and adipose tissue physiology is at least partly mediated through the activation of Gi/o-protein coupled receptors.

4.4 Discussion

The pathogenesis of obesity-related pathology is still only partly understood. Accumulating evidence, however, shows that the adipose tissue during obesity is a site of low grade inflammation. In the present study we showed that explants of human omental adipose tissue from obese patients produce substantial amounts of the most examined inflammatory mediators and that within this tissue various ATM-specific genes, like CD16, CD31, CD163 and MMP-9 are abundantly expressed, further supporting the concept that the human adipose tissue is a site of low grade inflammation.

Obesity is associated with increased infiltration of macrophages into human adipose tissue (29), which are identified, as we mentioned earlier, via specific ATM markers. Chemokines are crucial for the attraction of mononuclear cells from the circulation into adipose tissue (34;35). Herein we demonstrated that PA downregulates the production of various chemokines, cytokines and ATM markers. In addition, ATM markers were either not detected or very low in adipocytes compared to adipose tissue. Taking together, this implies that non-adipocyte cells, most likely ATM, contribute to the immunomodulatory effects of PA.

It has been recognized before that the colonic microbiota can influence both the propensity to as well as the course of obesity-related pathology. Mechanisms identified in this process are that different species of the microbiota

may differ in their capacity to ferment ingested food components and that the fermentation products may differentially effect mucosal immunology and thus influence host health in a paracrine fashion (6;8). In the present study we show, however, that the microbiota does act in trans, via the release of PA. Specific microbial species differ greatly in their capacity to produce PA from food. Since differences in the amount of PA produced will have implications for the immunological, lipogenic, adipogenic and insulin sensitivity effects of PA, our data show that the metabolism of the intestinal microbiota can have direct consequences on the physiology of distal adipose tissues.

These data correspond well with other studies that show that diets that are likely to enhance the production of PA by the microbiota correlate with lower incidence and better outcome of metabolic syndrome and other obesity-related diseases (32;33). Furthermore, our observation that the diminished inflammation induced in the adipose tissue explants by PA is also accompanied by improved expression of factors associated with lipogenesis, adipogenesis and insulin sensitivity, leads us to hypothesize that PA produced in the colon may have a direct beneficial effect on the adipose tissue. Thus we feel that PA may constitute an important novel link between the microbiota and the physiology of adipose tissue.

Two receptors for SCFA in general and PA in particular have been identified, GPRC41 and GPRC43. We observed the expression of these receptors in human adipose tissue (36), suggesting that the effects of PA on the adipose tissue might be mediated through these receptors. Support of this notion was found from our experiments that at least part of the responses to PA are sensitive to PTX, generally considered as a specific inhibitor of Gi/o proteins and hence suggesting that G-protein coupled receptors mediate the response to PA. In addition, genetic evidence exists that GPRC43 acts as an anti-inflammatory protein, because mice genetically deficient in active GPRC43 are prone to exaggerated inflammation in a

variety of murine models. Hence GPRC43 is a likely candidate to mediate at least part of the response to PA in human adipose tissue and selective agonists for this receptor may hold promise for combating metabolic syndrome. Experimentation addressing this notion is currently under progress in our laboratory.

4.5 References

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Propionate: a Candidate Metabolite to Link Colonic Metabolism to Human Adipose Tissue Inflammation

Chapter 5

Propionic Acid Exerts Anti-Inflammatory Effects on Human Macrophages: a Proteomics Approach

Sa'ad Al-Lahham, Desiree Weening, Han Roelofsen, Farhad Rezaee, Roel Vonk and Koen Venema

In preparation

Abstract

Introduction: Macrophages play a major role in the induction of inflammation, which is evidenced in a variety of modern diseases such as diabetes and inflammatory bowel diseases. The factors influencing macrophage-induced inflammation and subsequent pathology remain poorly understood. However, accumulating evidence suggests that the fermentation of dietary fiber by the intestinal microbiota appears to be protective. Short-chain fatty acids, e.g. propionic acid (PA), are the principal products of this fermentation. Therefore, we investigated the effects of PA on the secretome of human macrophages.

Materials and methods: THP-1-derived macrophages were incubated for 2 hours with various concentrations of lipopolysaccharide (LPS) alone or with a combination of LPS and PA. Stable isotope labeling by amino acids in cell culture (SILAC) was employed to investigate changes in macrophage protein production in response to PA treatment. SILAC results were validated by ELISA.

Results: In total 314 proteins were identified, 59 and 34 proteins were up-regulated and down-regulated respectively by PA treatment, of which 29 and 27 proteins were secreted respectively. The secreted down-regulated proteins belonged to various functional categories, among them inflammatory proteins (11 proteins). From these proteins, IL-8 and TNF-α, were validated by ELISA. Furthermore, ELISA showed that 6 other chemokines, which were below the limit of detection of SILAC, were also down-regulated.

Conclusion: The negative cross-talk between PA, which is produced by the microbiota in humans, and the macrophage inflammatory process provides a new paradigm in understanding the relation between the microbiota and macrophages-induced inflammation.

5.1 Introduction

A large body of evidence shows that tissue-resident macrophages (1) are a contributing factor to the pathogenesis of inflammation associated diseases, including obesity (2;3), insulin resistance, diabetes (4), cardiovascular diseases (5;6) and inflammatory bowel diseases (IBD) (7). The etiology of these diseases is complex and involves not only macrophages, but also other biological and environmental factors. It has become clear that the microbiota composition and metabolic activity is an important factor in this respect. It has been reported that these factors influence the development of obesity (8;9), systemic inflammation (10) and IBD (11). The mechanisms by which colonic microbiota composition can have such consequences on inflammation and perhaps macrophages remain, however, obscure at best.

Dietary fiber intake is associated with a reduction in chronic inflammation and in intestinal inflammatory pathogenesis (12-15). This may be due to the fermentation of dietary fibers by the microbiota. Short-chain fatty acids (SCFA), in particular acetic, propionic and butyric acid, are the major microbial metabolites that are produced. Indeed inflammatory and clinical parameters of inflammatory bowel disease patients have been improved by SCFA enema treatments (16) through the inhibition of macrophage activation (17). However, butyric acid was studied the most (18), while little is known about propionic acid (PA).

Recently, it has been shown that PA possesses anti-inflammatory properties on human neutrophils and mouse colonic organ cultures (19). In addition, we have shown (20) and unpublished data) that PA influences the physiology of human adipose tissue, which is the principal site for obesity-induced inflammation. An important outcome of these experiments was that PA inhibited the production of inflammatory markers including adipose tissue macrophage (ATM) related markers. However, the effect of PA on macrophages has not been investigated yet. Therefore, we investigated the influence of PA on the secretome of macrophages.

To achieve our aim we employed a proteomics technique (SILAC) to investigate the relative quantity of protein abundance in the secretome of treated (PA + LPS) versus control (LPS alone) macrophages and the outcome was further validated by ELISA.

5.2 Materials and methods

5.2.1 Materials

RPMI-1640 media, light L-[$^{12}C_6$] arginine (L-Arg) and L-[$^{12}C_6$] lysine (L-Lys) isotopes, regular and dialyzed fetal bovine serum (FBS), proline, leucine, penicillin and streptomycin antibiotic mix, phorbol myristate acetate (PMA), PA, LPS, trifluoroacetic acid (TFA), dithiothreitol (DTT) and ammonium bicarbonate were purchased from Sigma (Zwijndrecht, The Netherlands), heavy L-[$^{13}C_6$] Arg and L-[$^{13}C_6$] Lys isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), SimplyBlue safe staining gels and MOPS buffer from Invitrogen (Breda, The Netherlands), carbamidomethylation and iodoacetamide from GE Health Care (Hoevelaken, the Netherlands), trypsin from Promega (Leiden, the Netherlands), acetonitrile (ACN) from Biosolve (Valkenswaard, the Netherlands) and formic acid (FA) from Merck (Schiphol-Rijk, the Netherlands).

5.2.2 THP-1 cell culture

Human THP-1 monocytes (kindly provided by the Department of Biochemistry, Academic Medical Centre Amsterdam) were maintained in RPMI-1640 phenol red-free media supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml

of streptomycin in humid atmosphere containing 5% CO_2 at 37ºC. To induce monocyte-macrophage differentiation, THP-1 cells were plated at a concentration of $5*10^5$ cells/ml and treated with 10 ng/ml PMA for 48 hours. THP-1-derived macrophages were treated for 2 hours, in triplicate, with various LPS concentrations alone (0.1, 1, 10, 100 and 1000 ng/ml) or in combination with various concentrations of PA (0.001, 0.01, 0.1, 1 and 10 mM).

5.2.3 SILAC setup

THP-1 cells were grown in RPMI media without standard L-Arg, L-Lys and phenol red and were divided into 2 experiments, SILAC-1 and SILAC-2. In SILAC-1, half of the THP-1 monocytes, which were considered control cells, were grown in medium supplemented with a light L-[$^{12}C_6$] Arg and L-[$^{12}C_6$] Lys isotopes while the other half of the cells, to be treated were grown in the same medium, but supplemented with the heavy L-[$^{13}C_6$] Arg and L-[$^{13}C_6$] Lys isotopes. In SILAC-2, cells were treated as in SILAC-1; however, the labels were switched. 10% dialyzed FBS, proline and leucine were added to the culture of both SILAC experiments. Cells were grown for 8 weeks in order to achieve near complete incorporation of the L-Arg and L-Lys heavy isotopes into proteins. Subsequently, cells were plated at a concentration of $5*10^5$ cells/ml and treated with 10 ng/ml PMA for 48 hours to differentiate them into macrophage-like cells. Differentiated cells were treated for 2 hours with 1 µg/mL LPS and 10 mM PA and were compared to the control cells, which were treated with 1 µg/ml LPS alone.

5.2.4 Protein Identification by LC-MS/MS

Supernatants from approximately $25*10^6$ THP-1-derived macrophages cells were harvested for the SILAC analysis. The cell-free supernatants were collected,

concentrated by ultrafiltration and protein concentrations measured by the Bradford assay. Equal amounts of total protein from the control (LPS-treated) and the stimulated (LPS+PA-treated) groups were pooled together and were fractionated by SDS-PAGE on a 4–12% bis-Tris gel with a MOPS buffer system according to the manufacturer's protocol (NuPAGE-Novex, Invitrogen). Proteins were separated for 50 min at 200 V, and staining of bands was performed overnight by SimplyBlue safe staining. Each lane was excised into 25 bands that were processed for tryptic digestion. Each band was cut into small pieces and stored at -20 °C until analysis. Then they were washed in ultrapure water and dehydrated in ACN. Each slice containing proteins was reduced with DTT (for 1 h at room temperature) and carbamidomethylation with iodoacetamide (45 min at room temperature in the dark). Gel pieces were subsequently washed with ultrapure water and ACN. Next, 5 µg/ml of modified trypsin (resistant to protolytic digestion) in 20 mM ammonium bicarbonate was added, and gel pieces were allowed to rehydrate on ice for 20 min. Digestion was carried out overnight at 37 °C. After that, the peptides were extracted by adding 0.1% TFA and pure ACN and then the extract was dried via speed vacuum and resuspended in 5%TFA and 50%ACN. The samples were dried again and resuspended in 0.1% FA to measure them by LC-MS/MS.

Separation of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase LC-MS/MS. The Agilent 1100 nanoflow/capillary LC system was equipped with a trapping column (5 x 0.3 mm, C18RP) (Dionex/LC Packings, Amsterdam, The Netherlands) and a nanocolumn (150 x 0.075 mm, C18PepMap) (Dionex/LC Packings). Peptides mixtures were injected into the trapping column at a flow rate of 20 µl/min (in 0.1% FA). After 5 min the trapping column was switched into the nanoflow system, and the trapped peptides were separated using the nanocolumn at a flow rate of 0.3µl/min in a linear gradient of elution from 95% A (0.1% FA) to 50% B (90% ACN, 0.1% FA) in 53 min

followed by an increase up to 80% B in 3 min. The eluting peptides were on-line electrosprayed into the QStar XL hybrid ESI quadrupole time-of-flight tandem mass spectrometer (ESI-qQTOF-MS/MS, Applied Biosystems, Framingham, MA; MDS Sciex, Concord, Ontario, Canada) provided with a nanospray source equipped with a Proxeon stainless steel needle (25-µm diameter). Typical values for emitter voltage were 2.25 kV in positive ion mode. Analyst QS 1.1 software (Applied Biosystems) was used for data acquisition in the positive ion mode typically with a selected mass range of 300–1500 *m*/*z*. Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to be 2 s. The three most abundant charged peptides above a 30-count threshold were selected for MS/MS and dynamically excluded for 30 s with 30-ppm mass tolerance.

5.2.5 Data Analysis

ProteinPilot 2.0 software (Applied Biosystems) was used to analyze the generated data. The search engine of the ProteinPilot makes use of the Paragon^{TM} algorithm (21). Search parameters such as modifications, substitutions, cleavage events, and mass tolerance are modeled with probabilities and did not require user-controlled settings. Options that were chosen within the program were: amino acid substitution (label, Arg +6, Lys +6); Cys alkylation with iodoacetamide; digestion with trypsin; gel-based identification; species, *Homo sapiens*; identification focus for biological modifications; thorough search. The software automatically detected the heavy/light peak pairs and calculated the heavy/light ratios based on the peak areas.

The majority of average protein ratios reported by Protein Pilot have a *P*-value (evaluating the statistical difference between the observed ratio and unity) and EF (error factor) for each protein ID. The EF term indicates the actual average values

lies between (reported ratio)/(EF) and (reported ratio) × (EF) at a 95% confidence. Only those protein matches having a *P*-value < 0.05, more specifically, if the protein was identified in the injection duplicates with $P < 0.05$, an average of the two ratios was calculated to represent its differential expression; otherwise, only the ratio with $P < 0.05$ was saved for that protein. Afterwards, we selected those with error factor (EF) < 2 as 'quantifiable entries' with a variation below 20% (22; 23). In addition, contaminant proteins that are present in the media but not produced by the cells were discarded. They were identified via switching the amino acid isotope labels as we mentioned earlier and consequently the ratios of contaminant proteins derived from SILAC-1 and 2 were hugely different.

Significantly changed proteins were analyzed by SecretomeP 2.0 (24) to identify the secreted proteins. Those proteins with a predicted signal peptide were considered as secreted via the classical pathway (endoplasmic reticulum/Golgi-dependent pathway). If no signal peptide was predicted but the NN score exceeded the value of 0.5, proteins were classified as secreted via the non-classical pathway. Proteins that did not match these criteria were considered as being intracellular.

Gene ontology analysis was performed using DAVID and STRING web tools and published knowledge. Default options were selected to analyze the protein function by the DAVID functional annotation clustering tool (25) and the threshold values of Enrichment Score (ES) was set at the default value (1.3). Functional analysis of the proteins was further studied by STRING with the high confidence setting. STRING is a database of known and predicted protein-protein interactions derived from genomic context, high throughput experiments, (conserved) co-expression, and published knowledge (26).

5.2.6 ELISA and multiplex

Secreted cytokine and chemokine proteins were measured by single- and multiplex-ELISA assays according to the manufacturer's description (R&D systems, Minneapolis, MN, USA and Bio-Rad, Hercules, CA, USA).

5.2.7 Relative Q-PCR

RNA isolation, cDNA synthesis and relative quantification of genes were performed as we described in our previous study (20). Primer pairs and probes for G-protein coupled receptor 41 (GPCR41) and 43 (GPCR43) were obtained from Applied Biosystems (ID numbers Hs00271131_s1 and Hs00271142_s1, respectively). The sequences of the primer pairs used for the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were (5'-GGTGAAGGTC GGAGTCAACG-3') and (5'-ACCATGTAGTTG AGGTCAATGAAGG-3'), while the sequence of the probe was (5'-CGCCTGGTC ACCAGGGCTGC-3').

Results

5.2.8 Dose-effect of PA treatment on the secretion of TNF-α by human macrophages

As a first step it was crucial to find out whether the PA receptors GPCR41 and GPCR43 were expressed by the THP-1-derived macrophages before investigating the effect(s) of PA on their secretome. As figure 1 illustrates, both of them were expressed and the quantity of GPCR43 mRNA was approximately 3 times higher than the quantity of GPCR41 mRNA ($P < 0.0001$). Their expression was significantly inhibited by approximately 21% and 50% respectively ($P < 0.01$) as a

response to PA treatment. Subsequently, THP-1-derived macrophages were treated for 2 hours, in triplicate, with various LPS concentrations alone (0.1, 1, 10, 100 and 1000 ng/ml) or in combination with various concentrations of PA (0.001, 0.01, 0.1, 1 and 10 mM) to determine the dose-response relationship of PA on the secretion of TNF-α. As expected, TNF-α secretion increased with increasing LPS concentrations (Figure 2; 0 mM PA). Addition of increasing concentrations of PA led to a dose-dependent reduction of TNF- α secretion. As demonstrated in figure 2F, average values of the relative quantities of all experiments were calculated and it was shown that 0.001, 0.01, 1.0 and 10.0 mM of PA inhibited significantly the secretion of TNF-α by approximately 22%, 31%, 53% and 79% respectively, while the effect of 0.1 mM PA was not significant.

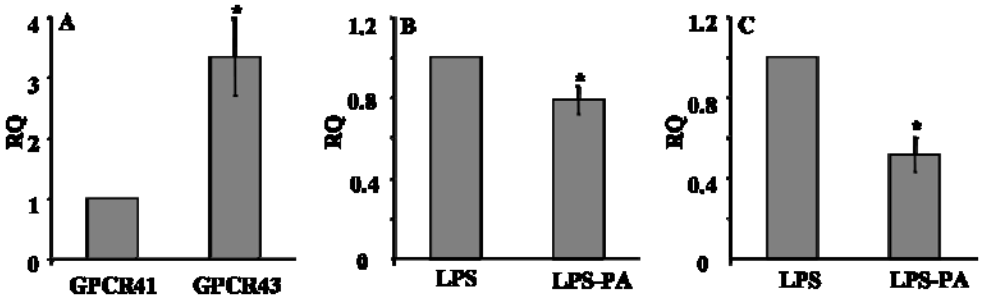

Figure 1: the basal levels of GPCR41 and GPCR43 mRNA expression (A) and their response to PA treatment (B and C). (A) The level of mRNA expression of GPCR43 in THP-1-derived macrophages was determined by RT-PCR and depicted as a relative quantity (RQ) compared to GPCR41 level. (B and C) THP-1-derived macrophages were incubated in triplicate with 1 μg/ml LPS alone or with a combination of 1 μg/ml LPS and 10 mM PA for 2 hours. Expression of GPCR41 (B) and GPCR43 (C) are expressed as RQ compared to their expression before incubation with LPS and/or PA. Error bars represent ±SD. **P* < 0.01 vs. control.

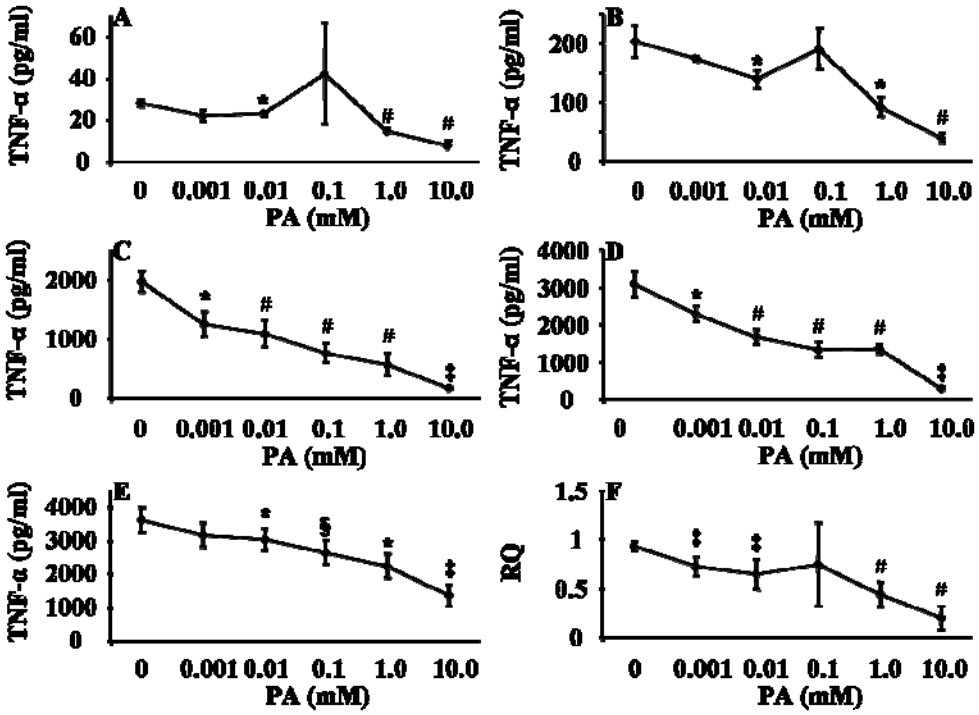

Figure 2: Dose-response curves of PA on TNF-α secretion from THP-1-derived macrophages. THP-1-derived macrophages were treated for 2 hours, in triplicate, with various concentrations of LPS 0.1 (A), 1 (B), 10 (C), 100 (D) and 1000 ng/ml (E) and each of these concentrations was combined with or without various concentrations of PA (0.001, 0.01, 0.1, 1 and 10 mM). F represents the averages of the magnitude of each effect that corresponds to each PA concentration occurring in the experiments of various LPS concentrations. The secreted quantity of TNF-α in the media was determined by ELISA and the results are depicted as absolute quantity (A-E) and as a RQ (F) compared to the control (LPS alone). Error bars represent ±SD. **P* < 0.05, ‡*P* < 0.01, #*P* < 0.001 and §*P* = 0.05 vs. control.

5.2.9 Identification and relative quantification of macrophage secreted proteins using SILAC

Based on the results above, we selected the extreme condition (1 µg/ml LPS-10 mM PA) to increase the detection limits of SILAC; since, it is known that the technique does not easily detect the low abundant proteins such as some cytokines and chemokines. Based on our criteria (see materials and methods for details) a total of 314 non-redundant proteins were identified from both SILAC experiments,

of which 243 proteins were overlapping (Table 1). It is known that PA has a mild effect (±25% - ±200%) on production of various parameters from various human cells and tissues, e.g. inflammatory parameters and adipokines production by colonocytes, neutrophils and adipose tissue (19;20); therefore, any protein with an average ratio either above 1.35 or below 0.65 can be considered showing a significant degree of up- or down-regulation compared with the mean (1.00). Figure 3 demonstrates the relative expression of all proteins and as summarized in Table 1 we found 59 and 34 proteins that were up- and down-regulated respectively. Later on, we predicted secreted proteins in the up- and down-regulated identified proteins based on secretomeP software. There were 29 and 27 secreted proteins up- and down-regulated, respectively. The list of those proteins is illustrated in Table 2.

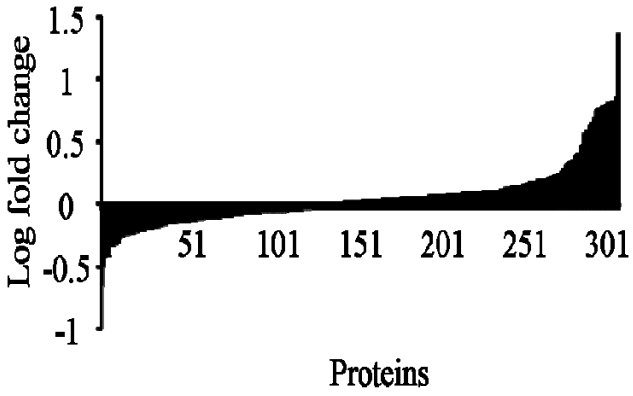

Figure 3: relative expression of all identified proteins. The 314 proteins were plotted as a logarithmic fold change. Positive and negative values indicate an up-regulation and down-regulation, respectively, in the presence of PA.

Table 1: Summary of the identified proteins in each data set.

	Number of the proteins	**Upregulated proteins**	**Downregulated proteins**
SILAC-1	305	56	32
SILAC-2	252	42	26
overlapping proteins	243	37	24
Total nonredundant proteins	314	59	34
secreted non-redundant proteins		29	27

Table 2: Differentially changed secreted-proteins.

Accession number	Name	Average of the fold change
P01375	Tumor necrosis factor (TNF)*	0.23
P10145	Interleukin-8 (IL8)*	0.32
O14773	Tripeptidyl-peptidase 1 (TPP-1)#	0.39
P07711	Cathepsin L1 (CTSL1)#	0.4
P07602	Proactivator polypeptide (PSAP)	0.46
P01033	Metalloproteinase inhibitor 1 (TIMP-1)#	0.47
P28799	Granulins (GRN)*	0.47
P12109	Collagen alpha-1(VI) chain (COL6A1)#	0.48
A6NC86	Uncharacterized protein ENSP00000244321	0.5
P36222	Chitinase-3-like protein 1 (CHI3L1)*,#	0.55
Q15084	Protein disulfide-isomerase A6 (PDIA6)	0.55
P07954	Fumarate hydratase, mitochondrial (FH)	0.56
Q9BS26	Thioredoxin domain-containing protein 4 (TXNDC4)	0.58
Q9Y240	C-type lectin domain family 11 member A (CLEC11A)*	0.58
O00754	Lysosomal alpha-mannosidase (MAN2B1)	0.58
P01024	Complement C3 (C3)*	0.59
P00746	Complement factor D (CFD)*	0.6
P01023	Alpha-2-macroglobulin (A2M)*,#	0.62
P14780	Matrix metalloproteinase-9 (MMP-9)*,#	0.62
P61916	Epididymal secretory protein E1 (NPC2)*	0.62
Q09028	Histone-binding protein RBBP4 (RBBP4)	0.63
P01034	Cystatin-C (CST3)#	0.63
P15144	Aminopeptidase N (ANPEP)*,#	0.63
P07339	Cathepsin D (CTSD)#	0.64
P07333	Macrophage colony-stimulating factor 1 receptor (CSF1R)*	0.64
P13489	Ribonuclease inhibitor (RNH1)	0.65
P42704	Leucine-rich PPR motif-containing protein, mitochondrial (LRPPRC)	0.65
P11021	78 kDa glucose-regulated protein	1.35
P84090	Enhancer of rudimentary homolog	1.39
P60981	Destrin	1.4
P61586	Transforming protein RhoA	1.45
P24666	Low molecular weight phosphotyrosine protein phosphatase	1.49
Q13185	Chromobox protein homolog 3	1.5
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	1.5
P23588	Eukaryotic translation initiation factor 4B	1.52
O60664	Mannose-6-phosphate receptor-binding protein 1	1.54

Q9NQR4	Nitrilase homolog 2	1.54
P29218	Inositol monophosphatase	1.55
P08708	40S ribosomal protein S17	1.57
Q9Y2S6	Coiled-coil domain-containing protein 72	1.61
O15511	Actin-related protein 2/3 complex subunit 5	1.66
Q15274	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	1.68
P08865	40S ribosomal protein SA	1.69
P63173	60S ribosomal protein L38	1.99
P14625	Endoplasmin	2.11
Q9HB71	Calcyclin-binding protein	2.13
P84077	ADP-ribosylation factor 1	2.42
Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	2.48
P62906	60S ribosomal protein L10a	2.83
P35268	60S ribosomal protein L22	2.87
P50502	Hsc70-interacting protein	3.58
P40306	Proteasome subunit beta type-10	4.59
P28070	Proteasome subunit beta type-4	6.12
P28062	Proteasome subunit beta type-8	6.43
P28072	Proteasome subunit beta type-6	6.46
P28065	Proteasome subunit beta type-9	7.02

*, proteins that belong to inflammatory proteins. #, proteins that belong to ECM remodeling proteins.

5.2.10 Gene ontology analysis

To assess differentially secreted proteins in the context of GO, we first applied the DAVID functional annotation clustering web tool to identify significantly over-represented functional annotations (Table 3). Up-regulated proteins can be mainly and significantly clustered into 4 major categories, proteasome subunits, cell cycle, protein translation and regulation of cytoskeleton organization. The percentages of up-regulated proteins belonging to these categories were 16%, 19%, 19% and 13%, respectively. While such proteins may have been derived from dying cells, it is possible that either they, or their breakdown products, may be actively secreted.

For example, proteasome subunits and ribosomal proteins are well-known intracellular proteins; however it was shown that they can be secreted (27;28). Regarding the secreted down-regulated proteins, 74%, 66%, 22%, 19%, 22% and 12% of these proteins significantly belonging to signal peptide, extracellular region, proteases, inflammatory response, regulation of cell proliferation and regulation of catabolic process, respectively (Table 3). Due to the fact that there is no single GO tool that can offer a complete picture for the functional annotations of a certain list of proteins (29), because each tool uses different public databases, we further analyzed the proteins by other tools, i.e. STRING, protein database (ExPASy), and by searching the published knowledge (Pubmed). However, because the functional annotations of down-regulated proteins had higher ES than of the up-regulated proteins (total ES = 24.63 and 11.99 respectively) and because they belong to more relevant physiological functions, the further analysis was focused on the secreted down-regulated proteins. Figure 4 demonstrates one major network determined by STRING, which is combined from 2 functional categories, inflammation and extracellular matrix (ECM) remodeling related proteins. This is in accordance to the DAVID output, however, based on all used tools the number of inflammation related proteins was increased to 11 proteins (Table 2), including, tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), granulins and complement C3, which were inhibited by approximately 77%, 68%, 53% and 41% respectively. Four of the inflammation related proteins were also shown (Table 2) to be involved in the ECM remodeling, i.e. aminopeptidase N ((ANPEP)) metalloproteinase 9 (MMP-9), alpha-2-macroglobulin (A2M) and chitinase-3-like protein 1 (CHI3L1), which were down-regulated by approximately 40%. Six more proteins belonged to ECM remodeling only, Tripeptidyl-peptidase 1, cathepsin L1 (CTSL1), metalloproteinase inhibitor 1, Collagen alpha-1(VI) chain, cystatin-C (CST3) and cathepsin D (CTSD), which were inhibited by approximately 61%, 60%, 53%, 52%, 37% and 36%, respectively (Table 2).

Table 3: GO analysis results.

Proteins response	**Functional annotation**	**Protein Nr.**	**Enrichment score**	***P*-value**	**Percentage***
upregulated	proteasomes subunits	5	4.06	1.63E-08	16%
	cell cycle	6	4.06	0.019	19%
	protein translation	6	2.34	7.49E-04	19%
	regulation of cytoskeleton organization	4	1.53	0.0027	13%
downregulated	signal peptide	20	9.73	1.26E-10	74%
	extracellular region	18	6.62	1.66E-08	67%
	Proteases	6	2.78	4.00E-04	22%
	inflammatory response	5	2.09	0.0025	19%
	regulation of cell proliferation	6	1.82	0.013	22%
	regulation of catabolic process	3	1.59	0.02	11%

* The percentage describes the percentage of proteins that significantly belong to a certain functional annotation

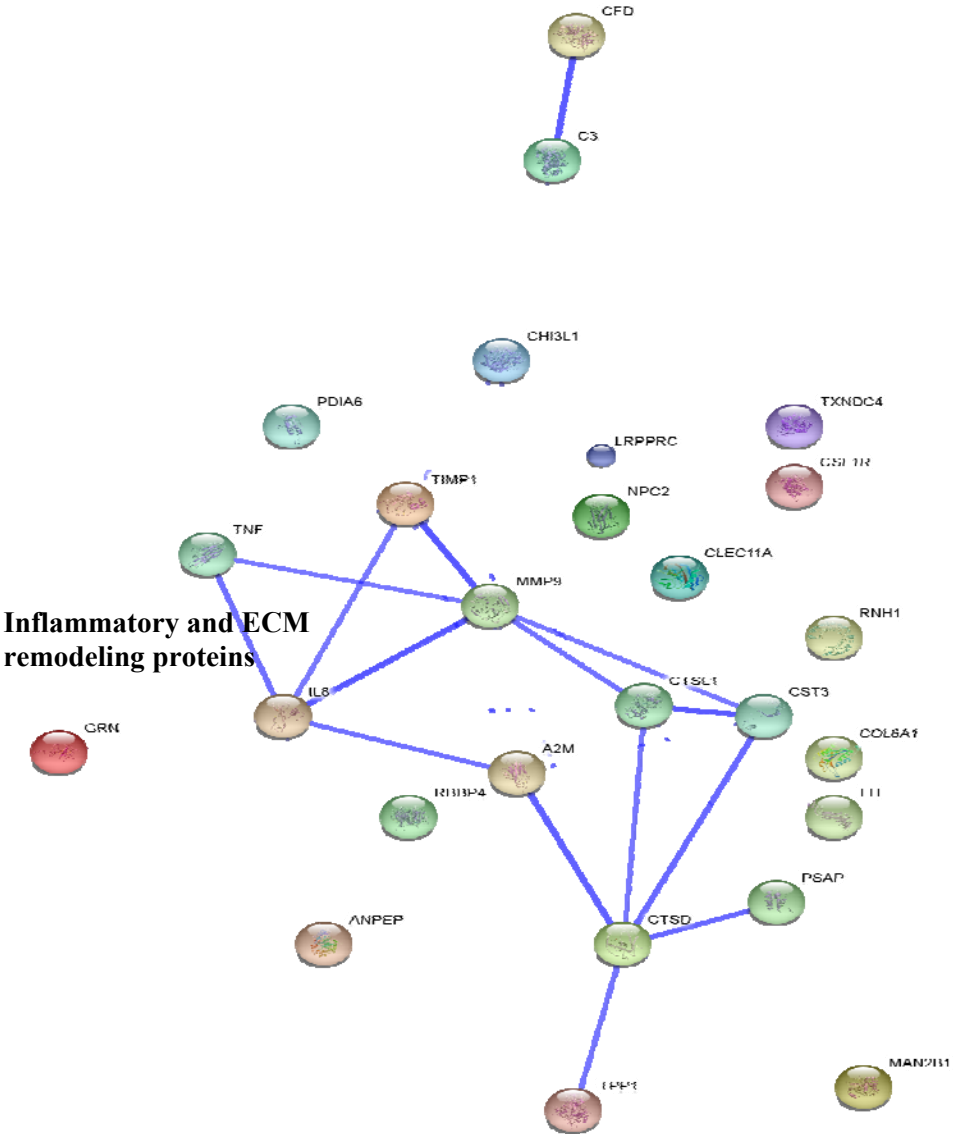

Figure 4: STRING analysis of the down-regulated proteins. Accession numbers of the secreted proteins that were down-regulated by PA treatment (Table 2) were submitted to STRING to determine their functional relationship. Stronger relationships are represented by thicker lines. One major network with interconnected proteins belonging to 2 functional categories, i.e. inflammatory and ECM remodeling, was identified. For protein abbreviations see Table 2.

5.2.11 PA possesses immunosuppressive properties

The above analysis strongly suggests that PA inhibits the secretion of pro-inflammatory parameters from THP-1-derived macrophages. To investigate this further, the effect on the secretion of TNF-α and IL-8 along with 11 other inflammatory parameters was measured via multiplex-ELISA on samples derived from the same and other 2 validation experiments. These parameters can be subdivided into 3 groups. The pro-inflammatory cytokines, i.e. interleukin-12 (IL-12) and TNF-α, the anti-inflammatory cytokines, i.e. interleukin-4 (IL-4), interleukin-10 (IL-10) and interleukin-13 (IL-13) and the pro-inflammatory chemokines, i.e. granulocyte-colony stimulating factor (G-CSF), IL-8, interferon-gamma-induced protein (IP-10), monocyte chemotactic protein-1 (MCP-1), Macrophage Inflammatory Proteins-1α and -1β (MIP-1α and MIP-1β) and regulated upon activation, normal T cell expressed and secreted (RANTES). As depicted in figure 5, PA significantly inhibited the secretion of TNF-α and unexpectedly IL-10 by approximately 67% and 77% respectively (P = 0.0003 and P = 0.00006, respectively). This has been noticed also by other studies (our unpublished data) and (19). It is known that TNF-α induces the production of IL-10, which acts as a negative feedback to inhibit the excessive production of TNF-α (19;30). This might contribute to the observed unexpected reduction of IL-10. Furthermore, PA had no effect on IL-4, while IL-12 and IL-13 were not detected. With respect to chemokines, PA significantly inhibited the secretion of G-CSF, IL-8, MCP-1, MIP-1α, MIP-1β and RANTES (but not IP-10) by approximately 46%, 44%, 47%, 40%, 38% and 25% respectively ($P < 0.05$).

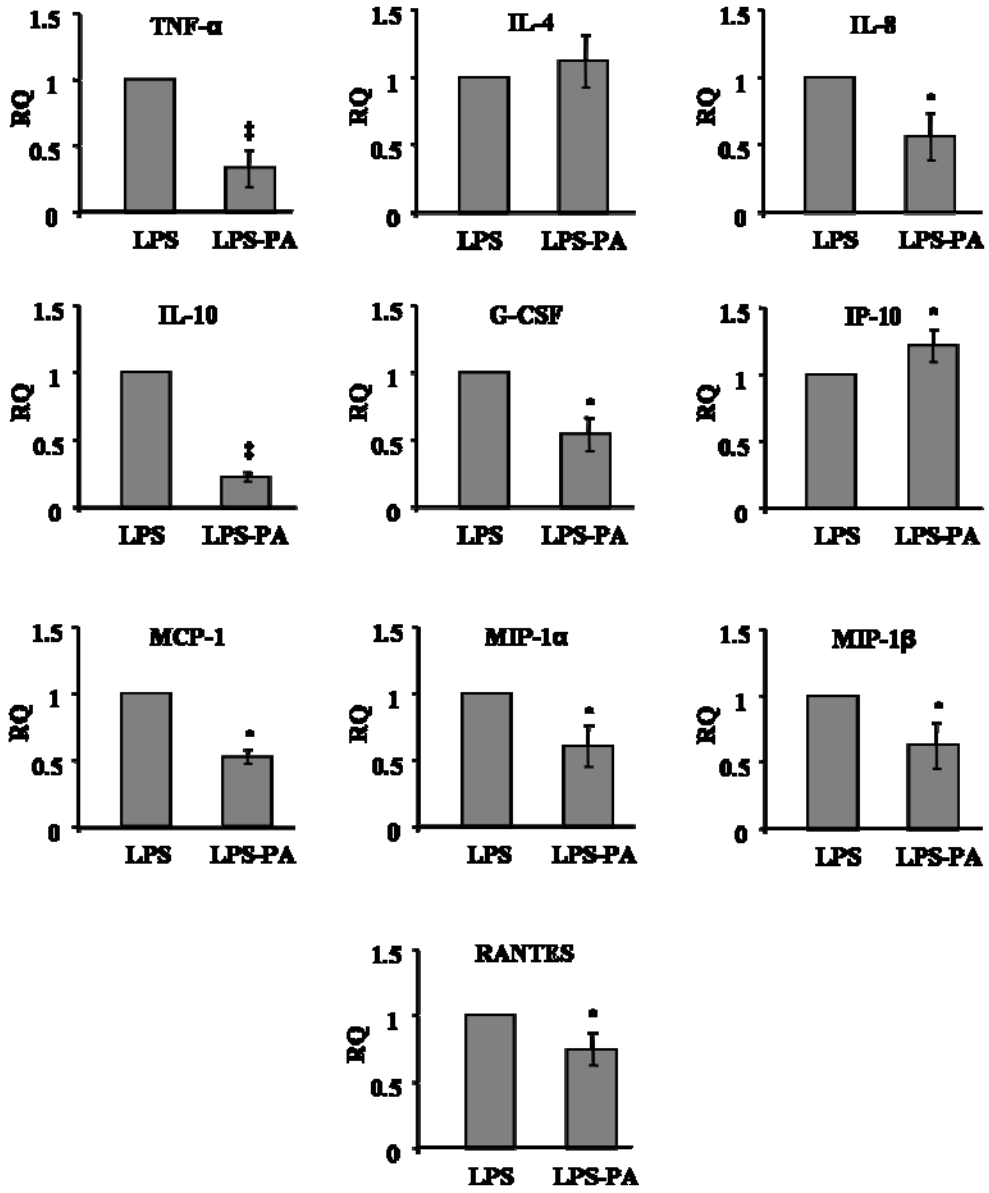

Figure 5: The influence of PA on the secretion of chemokines and cytokines by human macrophages. THP-1-derived macrophages were incubated in triplicate with 1 μg/ml LPS alone or with a combination of 1 μg/ml LPS and 10 mM PA for 2 hours. Secreted quantities of chemokines and cytokines in the media were determined by multiplex-ELISA. Results are depicted as relative quantities (RQ) compared to the control (with LPS alone). Error bars represent ±SD. *$P < 0.05$ and ‡$P < 0.001$ vs. control.

5.3 Discussion

In the human host, PA is mainly produced via colonic fermentation of dietary fiber by the microbiota where its quantity was reported to be 20 mmol/Kg (31). The majority of the PA produced in the colon is absorbed, passes the colonocytes and the viscera, and drains into the portal vein, where its quantity was reported to be 0.1 mM (31-33). PA can be partially metabolized by colonocytes; however, to what extent PA is utilized or interacts with visceral tissues has not been examined yet. Hence, various tissue resident macrophages, e.g. colonic, adipose and liver tissue macrophages, encounter and interact with different concentrations of PA. Although accumulating evidence suggests that PA exerts anti-inflammatory effects, its effect on macrophages has never been examined. Therefore, we investigated the influence of PA on the secretome of human macrophages by SILAC, a high throughput and robust assay, to quantify a broad range of proteins. We found in total 314 non-redundant proteins, of which 59 and 34 proteins were up-regulated and down-regulated, respectively by PA treatment. Of these, 29 and 27 proteins were secreted, respectively.

The secreted down-regulated proteins belonged to various functional categories, among them the inflammatory proteins. We demonstrated that 11 inflammation related proteins were downregulated and we validated two important proteins of these (TNF-α and IL-8) by ELISA. In addition, we analyzed the effect on other inflammatory parameters, which were under the limit of detection in the SILAC experiment, and we found that PA inhibits the secretion of 6 chemokines. These chemokines are known to be involved in the recruitment of macrophages into tissues, such as adipose tissue (34). Taken together, this suggests that

physiological concentrations of PA exert immunosuppressive properties on human macrophages, including the inhibition of the recruitment of further macrophages. Another interesting group of secreted down-regulated proteins is ECM remodeling proteins. We showed that 10 ECM remodeling proteins were down-regulated and 4 of them belong also to the inflammation related proteins. This is not surprising, since many ECM remodeling proteins are involved in leukocyte influx, chemokine and cytokine activation and formation of a chemokine gradient; for example it has been shown that MMP-9 activates IL-1β, IL-8 and TNF-α (35-37), suggesting that even though some cytokines and chemokines may still be secreted, PA still inhibits inflammation via inhibiting the activators (in this case ECM remodeling proteins) of these cytokines and chemokines.

Very recently, we have shown in one of our studies (unpublished data) that PA inhibited the production of various inflammatory parameters by human adipose tissue, including several cytokines, chemokines and markers of human adipose tissue macrophages (ATM). In addition, we found the expression of these ATM markers were not detected or very low in adipocytes compared to adipose tissue providing evidence that, at least partially, ATM contribute for the observed anti-inflammatory effects of PA, but not adipocytes. In the present study our data strongly support this finding.

It is recognized that the microbiota can influence the pathogenesis of various inflammatory related diseases. However, it is still not fully understood how the microbiota can influence these. One possible mechanism is that the microbiota influences host energy regulatory genes and/or proteins. Indeed, Backhed et al (38) found that the microbiota-induced deposition of triglycerides in adipocytes was mediated via the inhibition of the expression of the fasting induced adipocyte factor gene (Fiaf) in mice, which is an inhibitor for the lipoprotein lipase. However, these authors did not determine how the microbiota interacts with the host genes and via which mechanism the microbiota influences the expression of the host genes. In the current study we show that PA might be the mechanism by

which the microbiota may act on the host genes and thus providing a new paradigm in understanding the relation between microbiota and inflammation related diseases and suggesting that PA may have potential in preventing or treating inflammation related diseases.

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Propionate: a Candidate Metabolite to Link Colonic Metabolism to Human Adipose Tissue Inflammation

Chapter 6

Human primary adipocytes activate CD4+ T cells

Kees Meijer, Marcel de Vries, Sa'ad Al-Lahham, Marcel Bruinenberg, Desiree Weening, Martijn Dijkstra, Niels Kloosterhuis, Roelof Jan van der Leij, Han van der Want, Bart-Jan Kroesen, Roel Vonk and Farhad Rezaee

In preparation

Abstract

Background: Obesity promotes inflammation in adipose tissue (AT) and this is implicated in pathophysiological complications such as insulin resistance, type 2 diabetes and cardiovascular diseases. Although based on the classical hypothesis, necrotic AT adipocytes in obese subjects activate AT macrophages (ATM) that then lead to a sustained chronic inflammation in AT, to our knowledge, the link between human adipocytes and the source of inflammation in AT has not been studied systematically. So we decided to investigate human primary adipocytes alone to see whether they are able to prime an inflammatory state.

Methods and Results: Using mRNA expression arrays, human pre-adipocytes and adipocytes were shown to express the majority of known cytokines/chemokines and their receptors, MHC II molecule genes and 14 acute phase reactants including C-reactive protein. A specific multiplex ELISA approach revealed the expression of 50 cytokine/chemokine proteins by human adipocytes. Upon lipopolysaccharide stimulation**,** most of these adipocyte-associated cytokines/chemokines and immune cell modulating receptors were up-regulated. In a migration assay, human adipocyte-derived chemokines attracted significantly more CD4+ T cells than controls and the number of migrated CD4+ cells was doubled after treating the adipocytes with LPS. Neutralizing the monocyte chemoattractant protein-1 effect produced by adipocytes, reduced CD4+ migration by approximately 30%.

Conclusion: Human adipocytes express a large panel of cytokines/chemokines that are biologically functional. They are able to respond to an inflammatory stimulus and to activate CD4+ T cells independent of macrophages. This suggests that the primary event in the sequence leading to chronic inflammation in AT is a metabolic dysfunction in adipocytes, followed by production of immunological mediators by these adipocytes, which is then exacerbated by the activation and recruitment of certain immune cells.

6.1 Introduction

An imbalance or dysfunction of AT contributes to obesity-induced chronic inflammation, which in turn results in energy metabolism disorders such as insulin resistance (IR), type 2 diabetes, inflammation and cardiovascular diseases (1-11). Although the involvement of adipocytes in energy regulation is clear, little is known about their role in the occurrence of inflammation in AT, which is assumed to be important in the development of type 2 diabetes. Notwithstanding the fact that AT is recognized as an immune organ and the ATM appeared to be the major source of inflammation in AT (10-15), there has been no systematic study on the role of adipocytes in inflammation. We hypothesized that obesity led to adipocyte dysfunction, which in turn primes an inflammation state, although this priming is not due to adipose tissue-infiltrated macrophages. In contrast, inflamed AT adipocytes may promote the infiltration of ATM and change the macrophages with a resident character (anti-inflammatory macrophages called M2 macrophages) into migrated macrophages' phenotype (pro-inflammatory macrophages called M1 macrophages) (11) through a paracrine action, thereby establishing a vicious environment that exacerbates the inflammatory pattern in the AT and, in particular, in adipocytes.

To test our hypothesis, we investigated whether the adipocytes synthesize immune-associated components and if so, whether these adipocyte-expressed immune genes and proteins are biologically active and functional. In this study, we used the state-of-the-art proteomics, genomics and microscopy techniques.

6.2 Results and discussion

6.2.1 Differentiation of pre-adipocytes into adipocytes

To obtain a homogenous adipocyte fraction, human primary pre-adipocytes were differentiated into adipocytes (adipogenesis), which was morphologically monitored (Figure 1A-B). The efficiency of differentiation reached approximately 90-95% as assessed by morphology, indicating that the adipocytes could be considered as a homogenous fraction. In addition, Illumina BeadArray showed a significant up-regulation of 4 mRNA markers of adipocytes, i.e. adiponectin (ADIPOQ; 1000-fold), perilipin (PLIN; 500-fold), adipose triglyceride lipase (PNPLA2; 32-fold), and fatty acid binding protein 4 (FABP4; 128-fold). All these four genes are involved in lipid metabolism and the hydrolization of triglycerides (4;5;16;17) and were highly expressed in adipocytes compared to pre-adipocytes (Figure 1C). Perilipin (the hallmark of droplet fat), was also shown and confirmed at protein level by immunofluorescent confocal microscopy (CLSM) (Figure 1D).

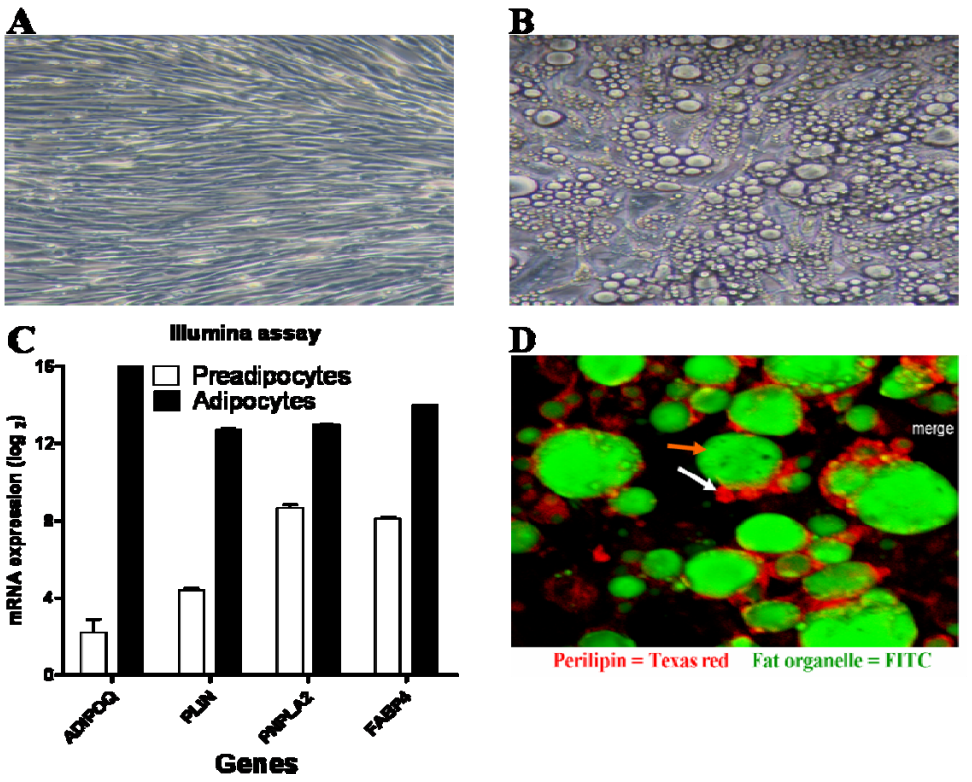
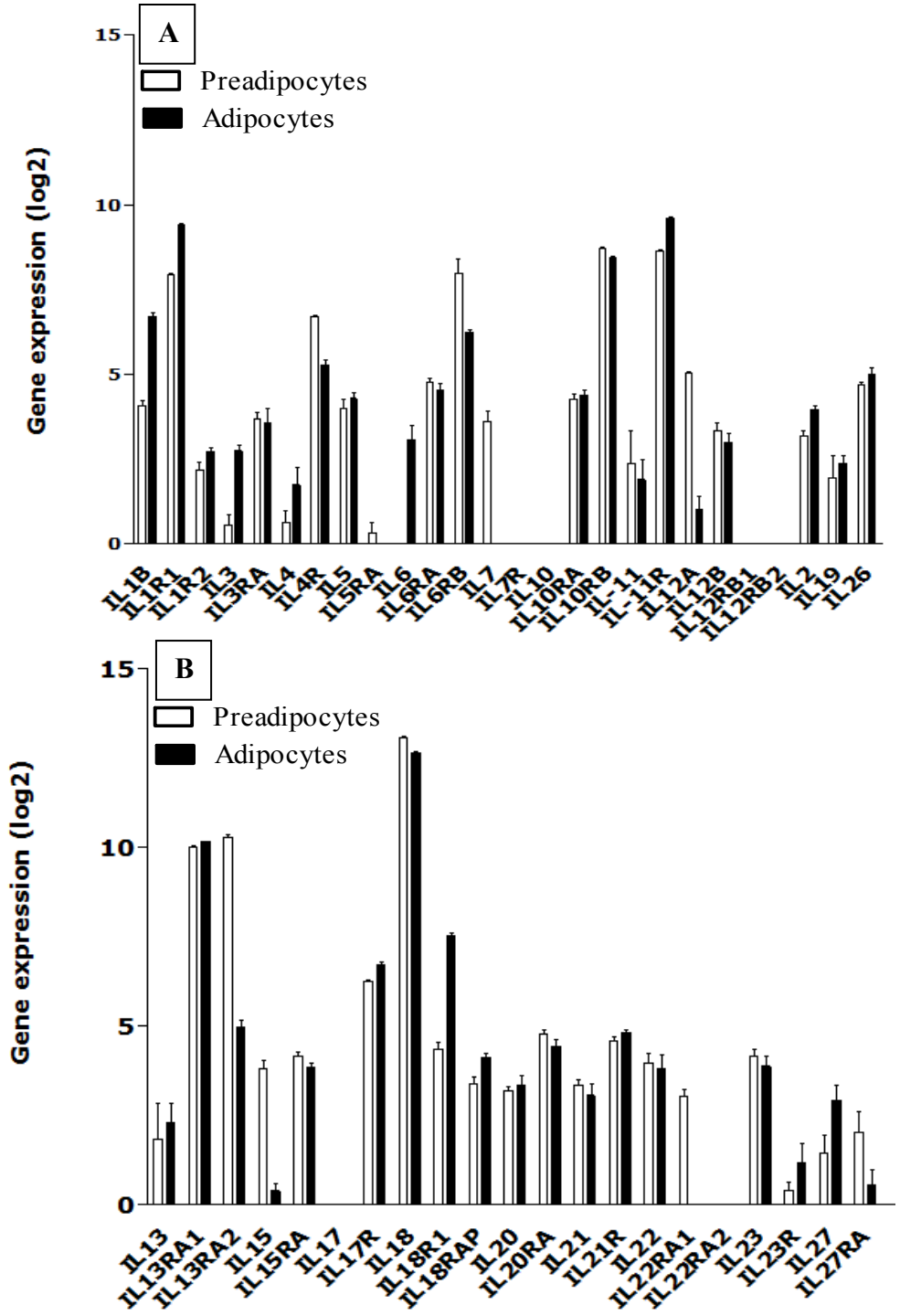


Figure 1: Differentiation of human pre-adipocytes into adipocytes. Panel A shows human pre-adipocytes (long, thin and flatted cells without detectable fat organelles). Panel B shows that differentiated fat cells (adipocytes) are spherical with differences in fat organelle size. Most space in adipocytes is occupied by fat organelles. Panel C displays differentiation markers for adipocytes. These markers are Adiponectin (AdipoQ), perilipin (PLIN), adipose triglyceride lipase (PNPLA2) and fatty acid binding protein (FABP4). Gene expression was expressed as log2 (e.g. the difference between pre-adipocytes and adipocytes for adiponectin expression is approximately 10, thus the true difference is 210 (=1024-fold) and this is shown on the y-axis). Confocal laser scan microscopy (CLSM, confocal microscopy) was used to validate and localize perilipin protein in human adipocytes as shown in panel D. Fat organelles were stained with FITC (green color), indicated by orange arrow, and Perilipin was visualized with Texas red (red color) shown by white arrow. Perilipin was localized at the surface of fat organelles.

6.2.2 Illumina (mRNA) BeadArray

After establishing our experimental set up, we determined immune-expressed genes in pre-adipocytes and adipocytes by Illumina BeadArray. Intriguingly, both

pre-adipocytes and adipocytes expressed the majority of known, immune-associated cytokine and chemokine genes (Figure 2A-C), either pro-inflammatory like TNF-α, IL-17, IL-19, CCL19 and CCL22 (6;9;11-13;15) or anti-inflammatory such as IL-4 and IL-13 (18;19). Based on the classical hypothesis, ATM is activated by triggers like lipopolysaccharide (LPS), which in turn produce pro-inflammatory chemokines and cytokines such as TNF-α, IL-6 and monocyte chemoattractant protein-1 (MCP-1; M1 macrophages). However, in our study we demonstrated that adipocytes can also contribute to the inflammation process.. Although no large differences were observed between pre-adipocytes and adipocytes with respect to expression of immune genes, IL-6 (pro-inflammatory) was only expressed in adipocytes, whereas IL-7 (a crucial role in T-cell homeostasis and could be considered as a new anti-inflammatory cytokine) was only expressed in pre-adipocytes (Figure 2A). Since human primary pre-adipocytes and adipocytes expressed substantial amounts of mRNA of many cytokines and chemokines, these immune genes seem to be an intrinsic property of this cell type. In the mRNA BeadArray we also found that both pre-adipocytes and adipocytes synthesize many cytokine and/or chemokine receptors (Figure 2A-C). Furthermore, a significant negative correlation was seen between cytokine expression levels and the expression of their corresponding receptors ($P<0.0007$; $r= -0.5$), except for IL-18, for which both the cytokine and its receptor mRNA were highly expressed (Figure 2D).


A
Preadipocytes
Adipocytes
Gene expression (log2)
15
10
5
0
IL1B
IL1R1
IL1R2
IL3
IL3RA
IL4
IL4R
IL5
IL5RA
IL6
IL6RA
IL6RB
IL7
IL7R
IL10
IL10RA
IL10RB
IL-11
IL-11R
IL12A
IL12B
IL12RB1
IL12RB2
IL2
IL19
IL26
B
Preadipocytes
Adipocytes
Gene expression (log2)
15
10
5
0
IL13
IL13RA1
IL13RA2
IL15
IL15RA
IL17
IL17R
IL18
IL18R1
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IL23
IL23R
IL27
IL27RA

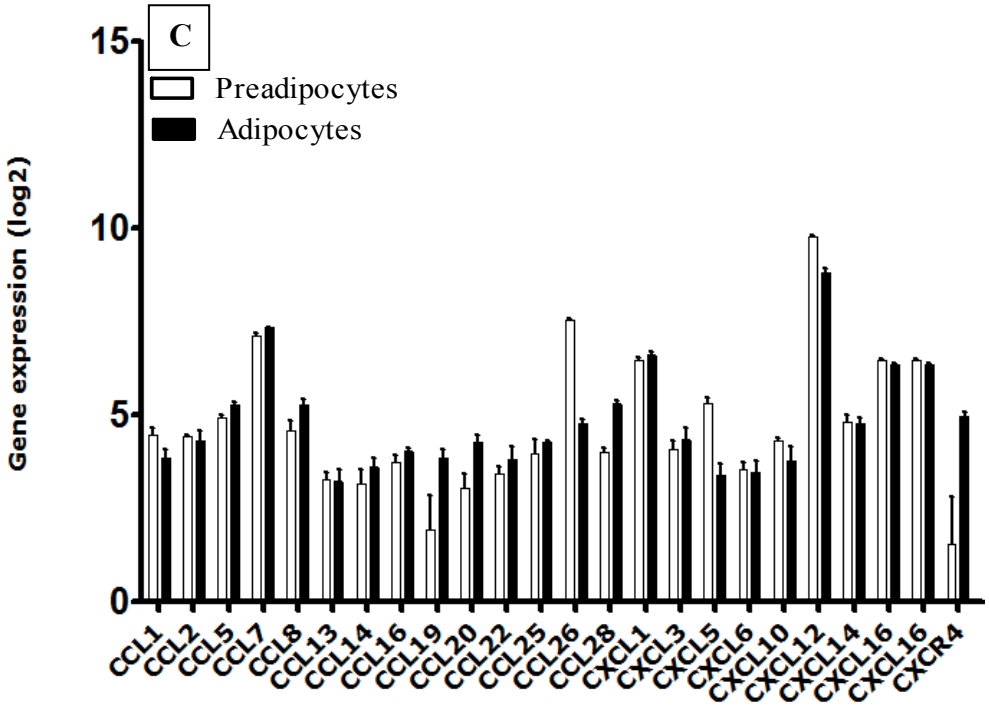


Figure 2A-C: Illumina gene profiling analysis of the cytokines and chemokines and their receptors expressed by the human adipocytes and preadipocytes. Illumina results were derived from two independent experiments. Each experiment was done in duplicate. Gene expression was expressed as log_2 and is shown on the y-axis.

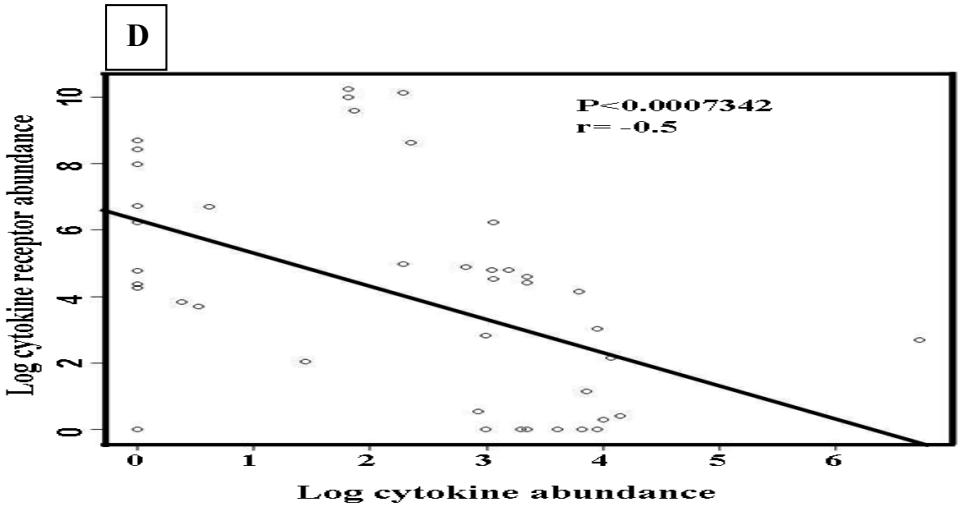
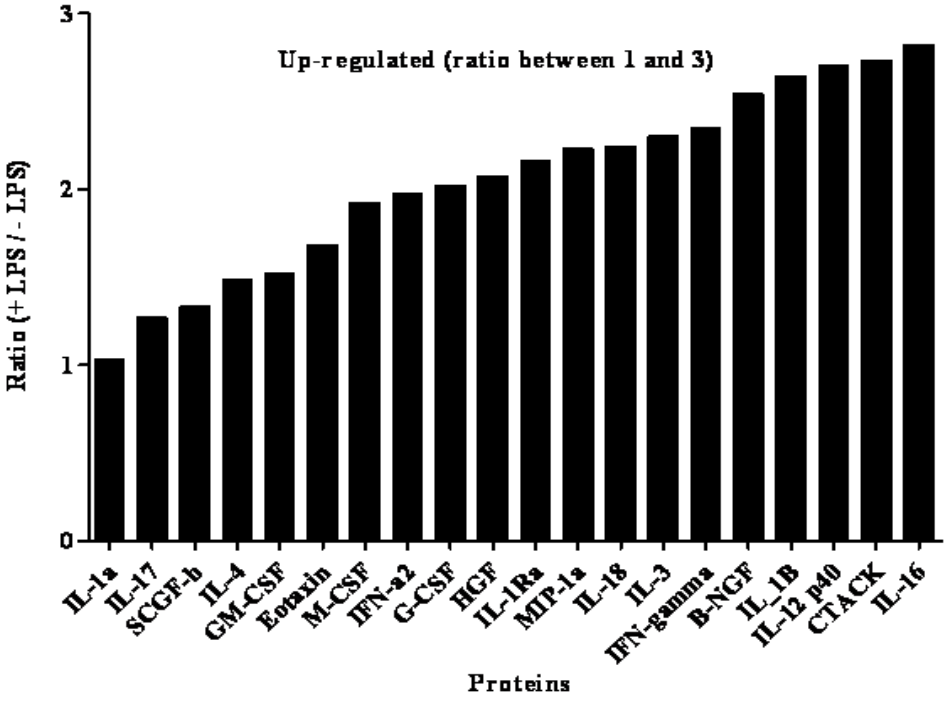


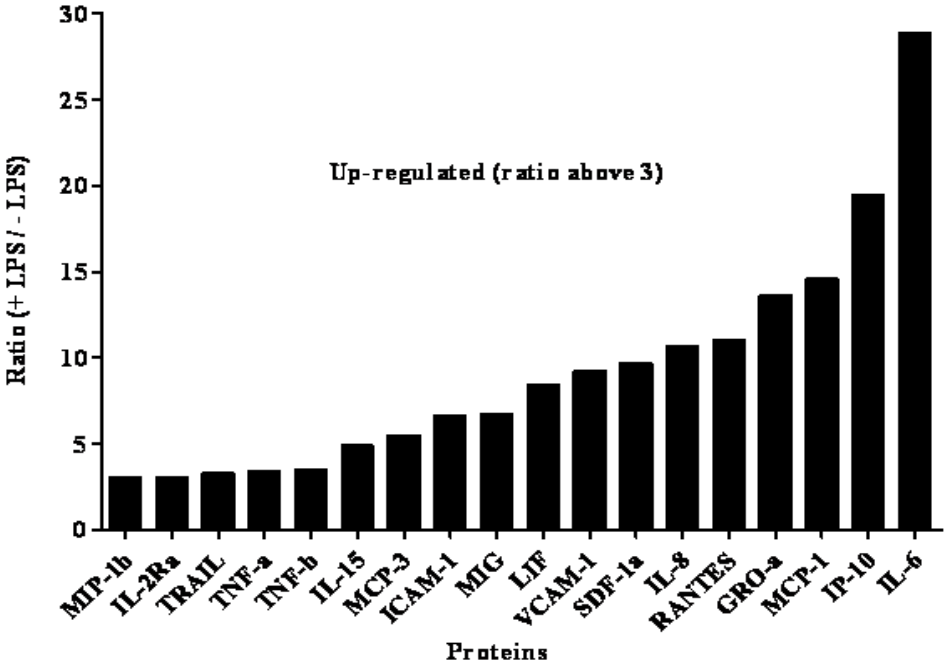
Figure 2D: the correlation between the cytokines and their receptors.

6.2.3 Proteomics, multiplex ELISA, confocal and immunoelectron microscopy

A proteomics approach was used to further validate the gene expression data. So far, all proteomics studies aiming at analyzing the adipocyte proteome have been performed in murine 3T3-L1 cells or in rat adipose cells (20) . A proteomic study of human primary adipocytes has not been reported before. Hence, a combination of different proteomics techniques, including LTQ-Orbitrap XT and Q-STAR, were used to analyze the production of cytokines and chemokines by human adipocytes. Although this approach led to the identification of approximately 1800 proteins, no cytokine or chemokine proteins were identified except for acute phase proteins. This is presumably due to their low abundance and the current mass spectrometry analyses/ sample processing is apparently not sensitive enough to detect such low abundances. Therefore, a very sensitive, multiplex ELISA with a panel of 50 cytokine and chemokine proteins was used to analyze whether these proteins are synthesized by human adipocytes. This resulted in the detection of all 50 cytokines and chemokines present in this panel in human adipocytes (Figure 2E-G). To investigate whether these cytokines and chemokines are biologically active, the differentiated adipocytes were stimulated with LPS (200 ng/ml with a final concentration of 1 µg for 24 h). Adipocytes treated with LPS led to the up-regulation (2E and 2F) of the majority of cytokines and chemokines, while a small fraction was down-regulated (Figure 2G). All of the up-regulated cytokine and chemokine proteins were pro-inflammatory (e.g. IL-6, TNF-α, MCP-1, IL-8) (Figure 2F), whereas a few down-regulated cytokines and chemokines were established anti-inflammatory (e.g. IL-7, IL-10 and IL-13) (Figure 2F and 2G). These results indicate that adipocyte-produced cytokines and chemokines are biologically active and might have a physiological relevance. Although no adipocyte-associated cytokines and chemokines were detected by the proteomics approach, this approach resulted in the identification of 11 known acute phase proteins (Table 1). Illumina BeadArray of human adipocytes confirmed the

expression of the majority of these acute phase proteins at gene level (Table 1). A combined analysis of proteomics and Illumina BeadArray led to the identification of 14 established (21-24) acute phase proteins including C-reactive protein (CRP) (Table 1), which is considered to be the most significant marker of inflammation. Both pre-adipocytes and adipocytes expressed the CRP gene, which was confirmed by RT-PCR analysis (data not shown). The fact that adipocytes synthesize CRP suggests that this gene was an intrinsic component of this cell. In addition, immunofluorescent confocal microscopy analysis using CRP-specific monoclonal antibodies (21) confirmed a substantial production of CRP at the protein level in adipocytes (Figure 3A-G). Immunofluorescent images were confirmed with immunoelectron microscopy using the same monoclonal antibody (Figure 3M-N). We focused our attention on the CRP because it plays an important role in host defense (immune-associated function) and elevated CRP levels are positively correlated to type 2 diabetes. CRP is also considered to be a prognostic marker in many diseases (22-24).


Up-regulated (ratio between 1 and 3)
Ratio (+ LPS / - LPS)
3
2
1
0
IL-1a
IL-17
SCGF-b
IL-4
GM-CSF
Eotaxin
M-CSF
IFN-a2
G-CSF
HGF
IL-1Ra
MIP-1a
IL-18
IL-3
IFN-gamma
B-NGF
IL_1B
IL-12 p40
CTACK
IL-16
Proteins


Up-regulated (ratio above 3)
Ratio (+ LPS / - LPS)
30
25
20
15
10
5
0
MIP-1b
IL-2Ra
TRAIL
TNF-a
TNF-b
IL-15
MCP-3
ICAM-1
MIG
LIF
VCAM-1
SDF-1a
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GRO-a
MCP-1
IP-10
IL-6
Proteins

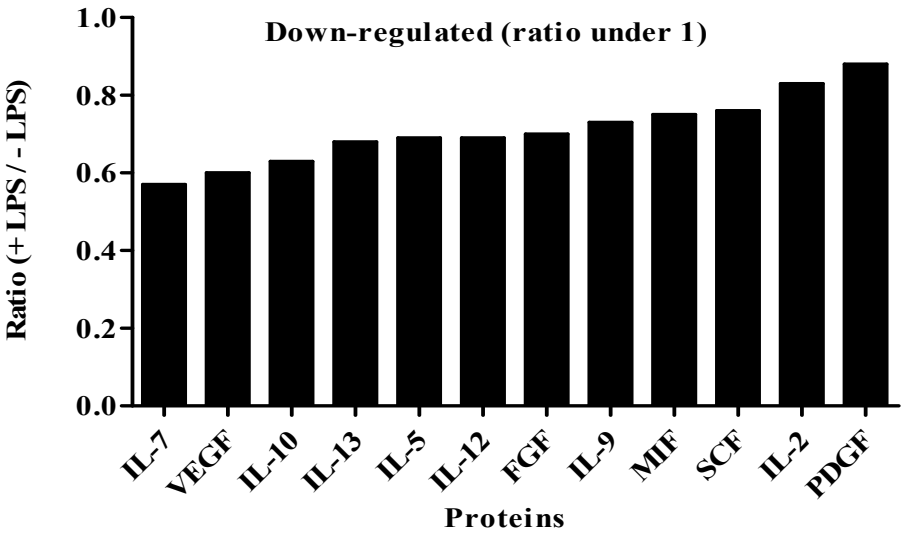


Figure 2E-F: LPS effect on the secretion of inflammatory parameter from adipocytes. (E) LPS upregulated the secretion of most inflammatory parameter by 1 to 3 folds and (F) above 3 folds and (G) down-regulated the rest of the parameters. The quantity of proteins was determined by multiplex ELISA protein BeadArray. Protein expression was expressed as the ratio of LPS-treated / untreated and is shown on the y-axis.

Table 1: The expression of acute phase genes and proteins by human primary adipocytes.

Protein Nr.	Gene Nr.	Protein name	Gene name	Secretion	PI	MW Da
P02765	112910	Alpha-2-HS-glycoprotein	AHSG	Yes	5,43	39325
P02768	113576	Serum albumin	ALB	Yes	5,92	69367
P02787	136191	Transferrin	TF	Yes	6,81	77050
P02751	2506872	Fibronectin	FN1	Yes	5,45	282607
P05121	129576	Plasminogen activator inhibitor-1	Serpin A1	Yes	6,68	45060
P35542	548885	serum amyloid A-4	SAA-4	Yes	9,27	14807
P01024	1,20E+08	Complement C3	C3	Yes	6,21	87148
P02794	120516	Ferritin heavy chain	FTH1	Yes	5,31	21226
P01019	113880	Angiotensiogen (SERPIN-A8)	AGT	Yes	5,87	53154
P05155	124096	Plasma protease C1 inhibitor	SERPING1	Yes	6,09	55154
P01011	112874	Alpha-a-antichymotrypsin (ACT)	SERPINA3	Yes	5,33	47651
P18510	124312	Interleukine-1 receptor antagonist	IL1RN	Yes	5,83	20055
P09919	4503079	Granulocytes-colony stimulating factor	G-CSF3	Yes	5,61	22293
P02741	5,60E+07	C-Reactive protein	C-RP	Yes	5,45	35039

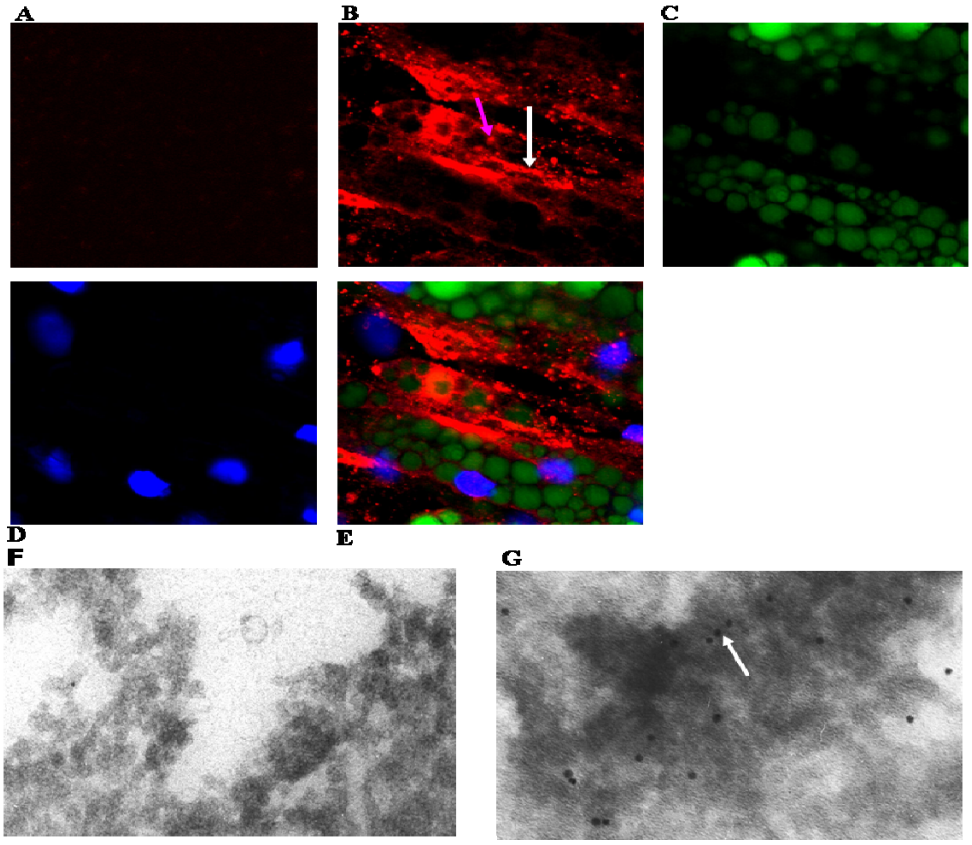


Figure 3A-G: Confocal microscopy analysis of C-reactive protein (CRP) in the human adipocytes. (A) Adipocytes were not incubated with the monoclonal antibody against human CRP (negative control). (B) Adipocytes were stained with human CRP antibody and bound antibodies were displayed with alexa 647 coupled goat anti-mouse (B and E; red color). Fat organelle (lipids) was visualized with FITC (C; green color), and adipocytes were stained with DAPI to detect nuclei (D; blue color). A merge of all three labels is shown in (E). White and red arrows indicate the presence of CRP on cytoplasm and plasma membrane, respectively. (F and G) immunoelectron microscopy images of the CRP in human adipocytes. (F) was a negative control, while (G) was labeled adipocytes with CRP antibody and bound antibodies were displayed with coupled goat anti-mouse 15 nm gold particles. White arrow shows one CRP particle.

6.2.4 Cell migration of CD4+ T cells, expression of MHC class II, and T cell co-stimulation genes by human adipocytes

Together, these results suggest that human primary pre-adipocytes and adipocytes exhibit immune cell-like behavior. In addition, as depicted in figure 2 F-G and 4 A-

B, adipocytes were found to express multiple receptors involved in T-cell (co)stimulation, especially upon treatment with LPS. These receptors included adhesion molecules, e.g. vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1-3 (ICAM-1-3), the ligand CD62L (CD34), CD36, CD44, CD47 and CD58; receptors involved in antigen presentation (MHC class II), e.g. C2TA (= CIITA), HLADM, HLADP, HLADQ and HLADR, CD1C-D, CD74 and CD4; receptors involved in T-cell co stimulation, e.g. B7.2 (CD86), CD2, CD38, CD40; as well as a significant number of cytokines and chemokines, e.g. MIP-1a, IFN-g, TRAIL, TNF-a, IL-8, RANTES, MCP-1, IP-10 and IL-6. It must be noted that many of these proteins have key functions in many aspects of the activation process leading to a cell-mediated immunity and, as such, are reminiscent of professional antigen-presenting cells (MHC II molecule) (25) . This observation led us to investigate whether human adipocytes exhibit an immune-cell function. We studied the role of human adipocytes in T-cell migration, which is an important requirement for allowing cell-mediated immunity. Since CD4+ T-cells are central to the regulation of cell-mediated immunity (25;26), we focused specifically on this cell population. We investigated the migration of CD4+ T cells towards human adipocytes (either treated with LPS or untreated) via the cell migration assay in a Boyden chamber (27) with a 5-µm-pore polyethylene terephthalate (PET) membrane (Millipore PET membrane insert for 12-wells,). CD4+ T cells were uploaded in the upper chamber (insert). As shown in Figure 4C, approximately 6000 CD4+ cells (assessed by counting of CD4+ in a Bürker-Türk chamber) (28) were migrated (after 10 h incubation) from the upper chamber towards the untreated human adipocytes (lower chamber), indicating that adipocyte-associated chemokines are functional. When human adipocytes were treated with LPS, the number of migrated CD4+ T cells approximately doubled (Figure 4C). We then investigated the role of the two most up-regulated chemokines (MCP-1 and IP-10) upon LPS stimulation (Figure 2F) in the migration of CD4+ T cells towards adipocytes. The effect of monocyte chemoattractant

protein-1 (MCP-1) and IP-10 were neutralized by adding of monoclonal anti-body against human MCP-1 and IP-10 (α-MCP and α-IP-10) to the adipocytes treated and untreated with LPS. When α-MCP-1 was added to the adipocytes treated with and without LPS, the migration of CD4+ T cells dropped significantly by approximately 30%. Although the neutralization of IP-10 exhibited a slight reduction of CD4+ migration, this decrease was not significant for this chemokine. In addition to two independent migration experiments with similar results, we also performed one migration assay with similar conditions but with an incubation time of 30 h. Intriguingly, we found that adipocytes treated with LPS increase CD4+ cell migration by approximately ten-fold in a time-dependent manner (Figure 4D). This could be due to a potentially increased concentration of adipocyte-associated chemokines after 30 h incubation with LPS.

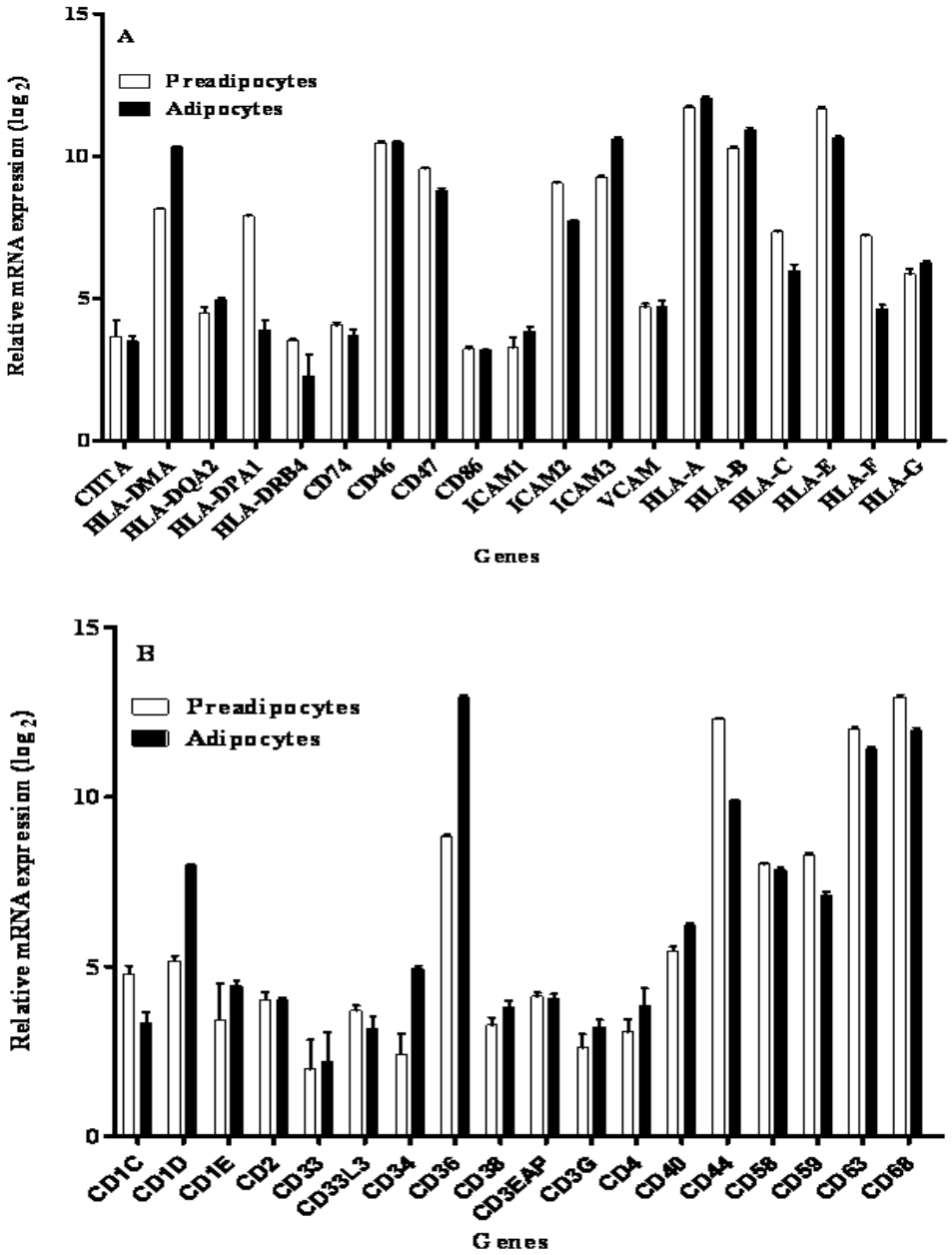
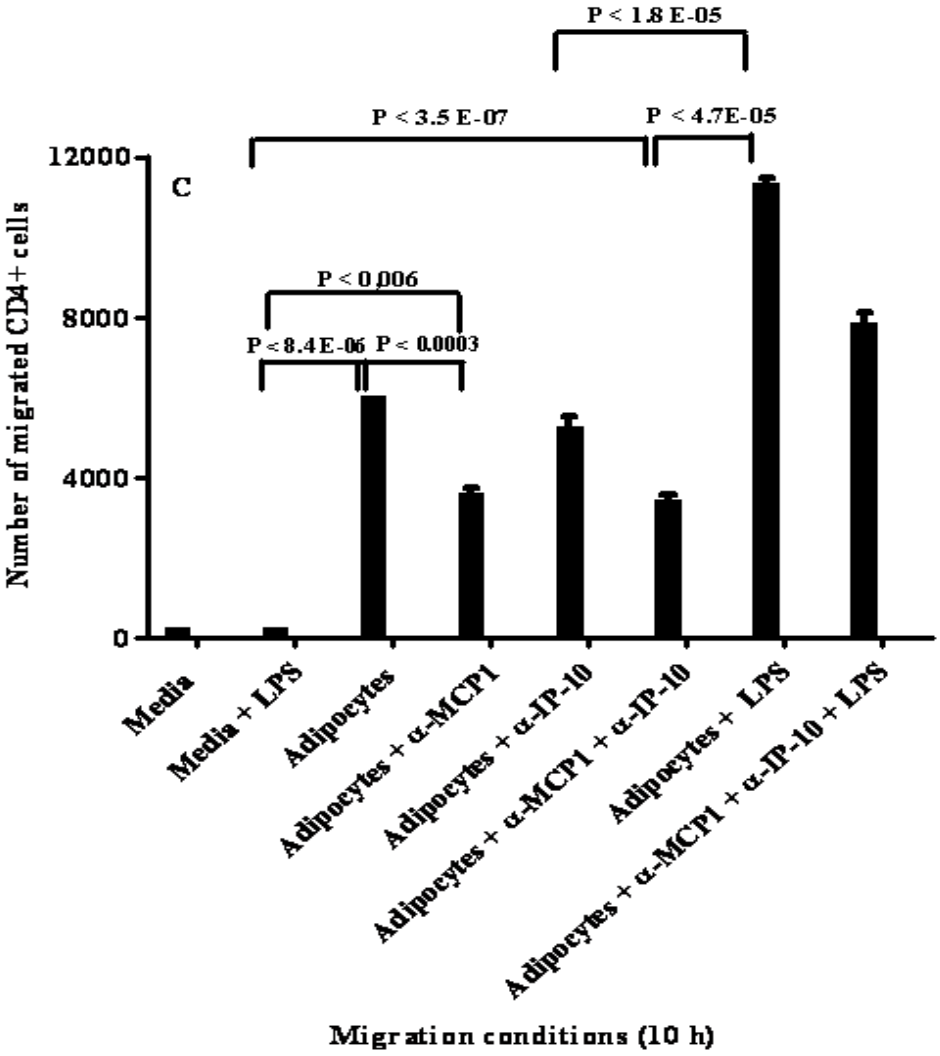


Figure 4A-B: The expression of the genes of MHC class II and receptors involved in (co)stimulation of T cells (A) and immune-associated cluster differentiation molecules (CD) (B), by human primary pre-adipocytes and adipocytes. Gene expression was given as log2 and shown on the y-axis. Illumina results were obtained from two independent experiments. Each experiment was done in duplicate.


P < 1.8 E-05
P < 3.5 E-07
P < 4.7E-05
C
12000
8000
4000
0
P < 0.006
P < 8.4 E-06
P < 0.0003
Number of migrated CD4+ cells
Media
Media + LPS
Adipocytes
Adipocytes + α-MCP1
Adipocytes + α-IP-10
Adipocytes + α-MCP1 + α-IP-10
Adipocytes + LPS
Adipocytes + α-MCP1 + α-IP-10 + LPS
Migration conditions (10 h)

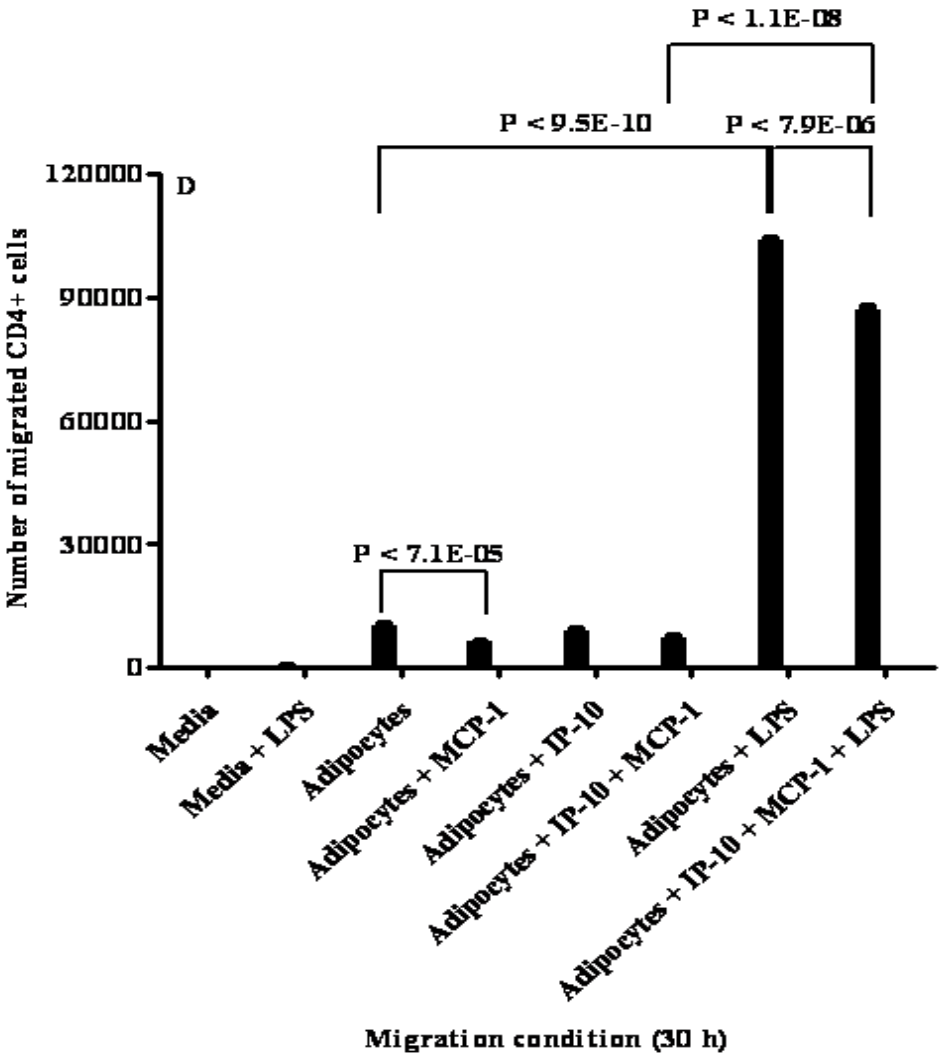


Figure 4C and D: the influence of the adipocytes and some of their chemokines on the migration of CD4+ T cells. To determine the role of the adipocytes and their chemokines in chemoattracting CD4+ T cells, adipocytes were grown on the surface of the lower compartment of the culture plate, where they attached to the surface. Then they were treated with or without LPS (200 ng/ml) and with or without antibodies against MCP-1 and IP-10 (2 µg/ml) to neutralize the effect of these two chemo-attractants. Purified and rested CD4+ T cells were added to the upper chamber. The cells were incubated at 37ºC for 10 h (C) or 30 h (D). The number of migrated cells was obtained using Bürker-Türk chamber. This experiment was repeated twice and each experiment was done in triplicate. Error bars represent the standard deviations. The p-value obtained from an independent two-tailed test samples (T-test). $P < 0.05$ was accepted as statistically significant.

6.3 Conclusion

We have revealed that adipocytes express cytokines, chemokines, multiple receptors, cell adhesion molecules and MHC class II family involved in the (co)stimulation of T cell activation. Since adipocyte-associated immune components (genes and proteins) responded to LPS stimulation, these immune components are biologically active with a physiological relevance in adipocytes. The functionality of these adipocyte-synthesized components was confirmed by assessing the role of adipocytes in immune cell migration, using CD4+ T-cells as responder cells. Cell migration assay showed that adipocytes are able to activate CD4+ T cells. Thus, adipocytes dysfunction promotes inflammation via its own cytokine and chemokine synthesis machinery and is not dependent on invading macrophages. This immune cell-like function of adipocytes could play a direct role in the etiology of insulin resistance and type 2 diabetes.

6.4 Acknowledgments

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Author Contributions FR designed, supervised the study and wrote the paper. FR and several authors performed the experiments. Authors also participated in the analyzing the data. Some authors also contributed to the editing of the paper.

Chapter 7

General Discussion, Concluding Remarks and Future Perspectives

7.1 General Discussion

Recently, it has been shown that colonic fermentation of dietary fiber/resistant starch has beneficial and protective effects against obesity and its associated disorders. Short-chain fatty acids (SCFA), in particular acetic acid, butyric acid, and propionic acid (PA) are the primary products of this fermentation. SCFA have recently attracted considerable attention, because of their impact on the host's health. However, most of the studies investigated the role of butyrate alone or mixed with other SCFA in colonic health and most of the few studies which considered PA alone were investigated in ruminants (**chapter-2**). While 90% of butyric acid is utilized by colonocytes and very little reaches the visceral tissues (1), the majority of the PA quantity produced in the colon (20 mmol/kg) (2) is absorbed, passes the colonocytes and the viscera, and drains into the portal vein. PA can be partially metabolized by the colonocytes; however, the quantity that is utilized by visceral tissues, e.g. omental adipose tissue, has not been examined yet. The quantity of PA in the portal vein in non-fasting humans was demonstrated to be approximately 0.1 mM (2-4). Together, it is fair to say that PA is a potential microbiota-derived metabolite to interact with the host physiology.

Indeed, important evidence for PA to act as a regulator of body-wide physiology exists. In 2003, three independent studies found that PA is a ligand for both G-protein coupled receptors 41 (GPCR41) and 43 (GPCR43) (5-7). GPCR41 and GPCR43, like other GPCRs, are linked to GTP-binding proteins (G-proteins). G-proteins serve as relay molecules functionally coupling the receptors to their downstream targets and include four major classes, namely Gs, Gi/o, Gq/11 and G12/13.

Taken altogether, one could envision that PA could constitute the elusive link between colonic-microbiota metabolism and the physiology of human adipose

tissue, including inflammation, insulin sensitivity, lipogenesis and adipogenesis, and consequently the whole body energy homeostasis, prompting us to investigate this hypothesis, which resulted in 4 studies, as discussed below.

7.1.1 The role of PA concentration

As eluded to above, PA can be found in various places in the human body. However, its physiological quantities are not the same in these locations. PA concentration in the lumen of the human colon is the highest and it is approximately 20 mmol/kg (2). As indicated before, the majority of PA is absorbed and passes the colonocytes and the viscera to reach the portal vein where 90% of PA is taken up by the liver (8) and the rest is transported to the peripheral blood. In humans, PA quantities in portal and peripheral blood were shown to be 100 µM and 6 µM, respectively (2;3;8-12). However, the quantity of PA that interacts with or is taken up by visceral (omental) adipose tissue is not investigated yet, most probably due to technical and ethical issues. As a consequence, the dose-effect relationship of PA on adipokine expression was determined in **chapter-3** *ex vivo* in adipose tissue. Leptin production was stimulated in adipose tissue by PA in the concentration of 1 and 3 mM, but not by 10 mM. On the other hand, resistin production was suppressed in adipose tissue by 3 and 10 mM PA. Consequently, 3 mM PA was used in studies described in **chapter-3 and -4**.

The concentrations of PA that were needed to show the observed results were relatively high (**chapter-3**). This is in agreement with earlier studies. Wajner et al (13) determined that PA concentrations above 2.5 mM were required to reduce the proliferation of activated lymphocytes. Moreover, Curi et al (14) found that PA concentrations equal or more than 3 mM showed remarkably high inhibition of lymphocyte proliferation. The requirement for high PA concentration could be due to the need for low pH values; since pH decreases as PA

concentration increases. However, in **chapter -3 and -4**, while the pH value of the media used for adipose tissue treatment remained neutral, PA was still effective. This is also in accordance with Brunkhorst et al (15) and Le Poul (5), who demonstrated that the activation of G-proteins by PA was not due to extracellular or intracellular pH.

7.1.2 PA influence on the production of adipokines by human adipose tissue

Adipose tissue is an essential endocrine tissue that produces various adipokines that act via autocrine, paracrine, endocrine and neuronal pathways to influence multiple physiological functions such as, satiety, energy balance, immunity and insulin sensitivity (16). Therefore, we determined in **chapter-3** the influence of PA on the production of few adipokines, i.e. leptin, resistin and adiponectin, on both mRNA and protein levels in omental adipose tissue derived from 28 women. We found that PA stimulated leptin, both on the mRNA and protein level, while it almost completely abolished resistin mRNA expression. On the other hand, adiponectin expression was not influenced. Induction of leptin by PA suggests that PA can contribute to the reduction of food intake and to the increase of energy expenditure, since leptin is a potent anorexigenic hormone (17). This notion is supported by human interventional studies, which showed that dietary supplementation of PA prolongs the satiety period (18;19). On the other hand, the inhibition of resistin suggests that PA may exert anti-inflammatory and insulin sensitizing effects, since it has been proposed that resistin is a pro-inflammatory and insulin resistance marker (20-23).

As different adipose tissue depots may vary in adipokine secretion patterns, we also investigated the influence of PA on the production of these

adipokines by human subcutaneous adipose tissue. The response of these adipokines in subcutaneous adipose tissue was similar to omental tissue, although it was less pronounced.

7.1.3 PA counteracts human adipose tissue inflammation

PA exerts anti-inflammatory effects in a variety of model systems (as summarized in **chapter-2**). Concomitantly, it has become evident that adipose tissue inflammation is a principal factor in the pathogenesis of obesity related diseases. We reasoned that obesity-related inflammation might be a target for the microbiota-produced PA. This hypothesis was supported by the inhibitory effect of PA on the production of the pro-inflammatory adipokine (resistin), which was observed in **chapter-3** and promoted us to further examine the effect of PA on the inflammation of adipose tissue in **chapter-4**. It was revealed that PA suppressed the release of the cytokine TNF-α and IL-10 and the chemokines, MIP-1α, MIP-1β, RANTES, G-CSF and IP-10 from adipose tissue. As chemokines are crucial for the attraction of mononuclear cells from the circulation into adipose tissue (24;25), our data suggests that PA could inhibit macrophages recruitment into adipose tissue. To further clarify this, we determined the effects of PA on the mRNA expression of ATM markers, CD16, CD31, CD163 and MMP-9 (26-29) by human adipose tissue. We found that the PA treatment significantly downregulated these markers. To clarify the role of human adipocytes we investigated the expression of ATM markers by adipocytes and we demonstrated that ATM markers were either not detected or very low in adipocytes compared to adipose tissue providing evidence that, at least partially, macrophages contribute to the anti-inflammatory effects of PA, and not adipocytes.

7.1.4 PA possesses anti-inflammatory effects on the human macrophages

The observation that the ATM contribute to the anti-inflammatory effects of PA, but not the adipocytes (**chapter-4**), persuaded us to further evaluate the role of the human macrophages. To accomplish that it was necessary to employ a powerful and robust assay, to quantify a broad range of proteins. Consequently, we employed the "stable isotope labeling by amino acids in cell culture" (SILAC) proteomic assay to investigate the relative quantity of protein abundance in the secretome of treated (PA + lipopolysaccharide [LPS]) versus untreated macrophages (LPS alone) (**chapter-5**). The results generated by SILAC were further validated by multiplex-ELISA assay. Also here, in the human macrophages, PA showed profound anti-inflammatory effects. This was demonstrated by the significant inhibition of the release of 17 inflammatory proteins, such as TNF-α, IL-8, granulins, complement subunits and 6 chemokines, providing more evidence that supports the notion that the inhibition of adipose tissue inflammation by PA treatment did not originate from adipocytes, but rather from the resident macrophages. Moreover, 10 extracellular matrix remodeling (ECM) proteins were downregulated, 4 of these proteins belonging to the earlier group of inflammatory proteins as well. ECM remodeling proteins are involved in leukocyte influx, chemokine and cytokine activation and formation of a chemokine gradient, for example it has been shown that MMP-9 activates IL-1β, IL-8 and TNF-α (30-32). These observations further support the finding that PA exerts anti-inflammatory effects on human macrophages.

7.1.5 PA positively influences factors associated with insulin sensitivity, lipogenesis and adipogenesis

The anti-inflammatory influence of PA on the adipose tissue may provide a mechanistic explanation as to the preventive influence of diets leading to the microbial production of such SCFA on the development of metabolic syndrome (33;34). If this notion is true, however, PA should negatively influence the associated-markers of metabolic syndrome. Thus, the effects of PA on several markers of adipogenesis, lipogenesis and insulin sensitivity were tested *ex vivo* in human adipose tissue. It appeared that human adipose tissue explants all reacted to such treatment by significant upregulation of the lipogenesis markers LPL and SREBP-1c (**chapter-4**), GLUT-4, a factor associated with insulin sensitivity (**chapter-4**), and the adiposity marker leptin (**chapter-3**), while it reduced the anti-adipogenesis markers pref-1 and resistin (**chapter-3 and -4**). These data are in agreement with mice studies, where it was shown that PA induced adipogenesis and inhibited lipolysis of mouse adipocytes (35;36). Taken together, we hypothesize that PA could induce the storage of fatty acids in adipocytes by inducing lipogenesis and adipogenesis,

7.1.6 Human primary adipocytes exhibit immune cell like behavior

Although our studies so far suggest that adipocytes do not contribute to the effects of PA, this does not necessarily imply that adipocytes have no role in the inflammatory process in human adipose tissue. To unravel this, human preadypocytes and adipocytes were studied in depth by genomics and proteomics approaches (**chapter-6**). It was shown, by illumina mRNA array, that both preadipocyte and adipocyte express various cytokines, chemokines, multiple

immune-related receptors, cell adhesion molecules and MHC class II family genes. The majority of these genes were shown, for the first time, to be expressed by preadipocytes and adipocytes. Interestingly, there were no large differences observed between preadipocytes and adipocytes with respect to the expression of these immune genes. Although proteomics approach identified approximately 1800 proteins, no inflammation-related proteins were detected except acute phase proteins. This is because the abundance of the inflammatory parameters was below the detection limits of our proteomics analyses. Therefore, we employed multiplex-ELISA assay and we found that adipocytes secrete all the examined (= 50) proteins. To determine whether adipocytes behave as immune cells we treated them with lipopolysaccharide and we found that they responded as immune cells by up-regulating the pro-inflammatory parameters and down-regulating the anti-inflammatory parameters. Finally, we examined whether adipocytes are involved in the initiation of the inflammation process. To achieve that, we assessed their ability to recruit T-cells after stimulating them by LPS, *in vitro,* and indeed T-cells were recruited. Thus, adipocyte dysfunction promotes inflammation via its own cytokine and chemokine synthesis machinery and this is not dependent on the invading macrophages. This immune cell-like function of adipocytes could play a direct role in the etiology of insulin resistance / type 2 diabetes.

7.1.7 The involvement of G-proteins coupled receptors

As we mentioned earlier, PA is a ligand for both GPCR41 and GPCR43, however, their expression by human adipose tissue was shown to be controversial. For example, Le Poul et al (5) showed that GPCR41 and GPCR43 were expressed in human adipose tissue, while Hong et al (35) showed it was not expressed in adipose tissue. Furthermore, the adipose tissue depot was not specified in these

studies. Our results show that both human adipose tissue depots (omental and subcutaneous) expressed GPCR41 and GPCR43 mRNA (**chapter-3**). Subcutaneous tissue expressed approximately 10-fold higher amounts of each receptor than omental (**chapter-3**). The presence of these receptors on human adipose tissue suggests that they might mediate the effects of PA on the adipose tissue. Support of this notion was found from our experiments (**chapter-3 and -4**) where we showed that the responses to PA were mediated via Gi/o-dependent and –independent signaling pathways. Gq-protein could contribute to the observed Gi/o-independent signaling pathway, since GPCR43 was shown to be coupled to both Gi/o- and Gq-proteins (5-7). In addition, genetic evidence exists that GPCR43 acts as an anti-inflammatory receptor (37). Therefore, GPCR43 is more likely to be a candidate receptor to mediate the anti-inflammatory effects of PA and selective agonists for this receptor may hold promise for combating metabolic syndrome.

7.2 Concluding remarks

In the present thesis, we demonstrate that PA exerts pronounced anti-inflammatory properties on human adipose tissue and macrophages *ex vivo* via Gi/o-dependent and independent signal transduction pathways. Furthermore, we show that PA treatment improves markers which could be linked to insulin sensitivity, lipogenesis and adipogenesis. Thus, we provide for the first time evidence that microbiological metabolites can influence the physiology of the human adipose tissue.

It is well known that the microbiota can influence the development of obesity and its associated pathogenesis. Very recently, it was found in mice that the microbiota induced the deposition of triglycerides in adipocytes via the inhibition of the expression of the fasting induced adipocyte factor gene (Fiaf) (an inhibitor for the lipoprotein lipase) (38). However, these authors did not determine how the

microbiota interacts and influences the expression of the host genes. In the current dissertation we show that PA might be the mechanism by which the microbiota may act on the host genes and thus providing a new paradigm in understanding the relation between microbiota and obesity and its related pathogenesis. Furthermore, it is tempting to suggest that the modulation of PA quantity through dietary manipulation and pre- and probiotics or supplementation of PA may have potential application in preventing obesity, suppressing low grade inflammation and protecting against its associated diseases, such as insulin resistance, type-2 diabetes and cardiovascular diseases.

7.3 Future perspectives

Our data lead us to speculate that the observed stimulation of lipogenesis and adipogenesis processes result in the clearance or redistribution of fatty acids. This would lead to the observed inhibition of the production of chemokines and would consequently lead to the inhibition of the recruitment of macrophages to adipose tissue. This all could lead to the observed insulin sensitivity improvement. In addition, we speculate that the stimulation of lipogenesis and adipogenesis cause the observed induction of leptin, since it is a marker of adiposity. This induction may lead to prolongation of satiety and inhibition of food intake. However, these speculations need to be further investigated in the future, preferably in human interventional studies.

PA mediates its signal via Gi/o-dependent and -independent signal transduction. However, many questions still remain unanswered and await further research. For example, why are there two GPCR receptors for PA and how do they interact with each other to convey PA signaling? Is the function of GPCR41 different from that of GPCR43, and if not, then why are these 2 receptors

expressed, in some cases, in the same cell type (e.g. adipocytes)? Answering these questions would be promising in terms of discovering or creating new ligands that may result in segregated effects of each individual receptor, with different outcomes. Finally, the potency for PA is low and PA receptors need supraphysiological concentrations of PA in peripheral blood to mediate its effects. This raises the question whether GPCR41 and GPCR43 are the right receptors for PA under normal physiological conditions in peripheral blood. It is possible that PA as well as other SCFA act as surrogate agonists rather than endogenous agonists for GPCR41 and GPCR43, or it is possible that these receptors are only activated in certain pathological situations when the PA concentration is unusually high, such as gingival inflammation and propionic acidemia. This suggests the necessity to screen for stronger agonists that can be used in the future instead of PA or may be combining PA with other SCFA, for instance acetate. Another interesting combination would be PA and thiazolidinediones, although they are not ligands for PA receptors. Thiazolidinediones inhibit low grade inflammation and improve pathological parameters of type-2 diabetes; however, they lead to an increase in body weight. It is suggested that the inhibition of leptin by thiazolidinediones contributes to the increase in body weight. To overcome this, it is essential not only to prevent the inhibition of leptin production, but rather to induce it. Therefore, combining PA with thiazolidinediones could prevent the increase in body weight simultaneously to the improvement of pathological parameters of type-2 diabetes.

7.4 References

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Propionate: a Candidate Metabolite to Link Colonic Metabolism to Human Adipose Tissue Inflammation

Summary

Summary

Obesity and its associated low-grade inflammation and pathological consequences, including insulin resistance, type-2 diabetes and cardiovascular diseases, pose a great challenge to the health systems nowadays. Adipose tissue is a primary site of obesity-induced inflammation which is emerging as an important biological contributor to the obesity-related diseases. Recently, it has become clear that life style factors, including nutrition, are as important as the biological factors in this respect. A large body of research indicates that dietary fiber appears to be protective. Moreover, accumulating evidence shows that the composition of the colonic microbiota is an important factor. However, the mechanisms by which they influence the development of obesity and its related pathophysiology remain, however, obscure at best.

Fermentation of dietary fiber by the colonic microbiota is the primary source for the production of short-chain fatty acids (SCFA), in particular propionic acid (PA). Therefore, one could envision that PA could constitute the elusive link between colonic-microbiota metabolism and the physiology of human adipose tissue. We investigated the influence of PA on the production of various markers of physiologically relevant functions by human adipose tissue *ex vivo*. It appeared that PA suppressed the production of various cytokines, chemokines and the adipose tissue macrophages (ATM) markers from adipose tissue. Moreover, it positively influenced the production of the markers associated with insulin sensitivity, lipogenesis and adipogenesis. This suggests that PA possesses anti-inflammatory effects that provides a mechanistic explanation to the observed improvement of insulin sensitivity, lipogenesis and adipogenesis and consequently

to the preventive influence of the dietary fiber on the development of the metabolic syndrome.

We also determined the contribution of the adipocytes and macrophages to the observed anti-inflammatory effects of PA. We demonstrated that ATM markers were either not detected or very low in adipocytes compared to adipose tissue. This implies that non-adipocyte cells, most likely ATM, contribute to immunosuppressive effects of PA. To evaluate further the role of macrophages, the effect of PA on the secretome of human macrophages was investigated via a quantitative proteomics approach. PA showed again overall anti-inflammatory properties, which were demonstrated by the significant inhibition of the release of 21 inflammatory proteins from the human macrophages.

Although we suggested that adipocytes do not contribute to the effects of PA we found that both preadipocytes and adipocytes produce various inflammatory parameters. In addition, LPS-stimulated adipocytes induced and reduced the production of the examined pro-inflammatory and anti-inflammatory parameters, respectively, and they were shown to recruit CD+4 T-cells. This implies that adipocytes exhibit an immune cell like behavior and they contribute for the initiation of the inflammation that occurred in the human adipose tissue independent of the ATM.

As PA is a ligand for both G-protein coupled receptors 41 and 43 (GPCR41 and GPCR43) and as both receptors activate the relay molecule Gi/o-protein, the last aim of our thesis was to unravel the role of Gi/o-protein in mediating the effects of PA in human adipose tissue explants. It was revealed that the response of all examined factors to PA treatment in human adipose tissue was either Gi/o-dependent or –independent signal transduction.

The results presented in this dissertation provide a new paradigm in understanding the relation between the colonic metabolism and adipose tissue physiology. Furthermore, our data suggests that the modulation of PA quantity may have potential application in suppressing low grade inflammation and

protecting from its associated diseases, such as insulin resistance, type-2 diabetes and cardiovascular diseases.

Nederlandse Samenvatting

Samenvatting

Obesitas en de daaraan gerelateerde ziektes zoals metabool syndroom, type 2 diabetes en hart en vaatziekten hebben grote consequenties voor de hedendaagse gezondheidszorg. Vetweefsel speelt een sleutelrol in de obesitas gerelateerde ontsteking, dat tegenwoordig wordt gezien als een belangrijke biologische factor in de obesitas geassocieerde ziektes. Recent is duidelijk geworden dat ook life-style factoren zoals voeding een zeer belangrijke rol spelen. Veel wetenschappelijke gegevens geven aan dat voedingsvezels een beschermende rol spelen. Ook worden steeds meer aanwijzingen gevonden dat bepaalde bacteriën in de dikke darm een belangrijke rol spelen. Echter, het onderliggende mechanisme is nog steeds onbekend. Fermentatie van voedingsvezels door bacteriën in de dikke darm is de belangrijkste bron van korte keten vetzuren (kkv), waaronder propionzuur. Wij hebben de hypothese ontwikkeld dat propionzuur een rol speelt in de relatie tussen het metabolisme in de dikke darm en de ontsteking van het vetweefsel. Wij hebben het effect van propionzuur onderzocht op de verschillende functies van het humane vetweefsel ex vivo.

Het bleek dat propionzuur de productie van diverse ontstekingsstoffen (cytokines, chemokines) en merkers van macrofagen (betrokken bij ontstekingen) kon remmen. Bovendien werden processen gestimuleerd die een positief effect kunnen hebben bij de ontwikkeling van obesitas geassocieerde ziektes (bv. insuline gevoeligheid). Dit suggereert dat propionzuur een anti-inflammatoir effect kan hebben dat de beschermende effecten van voedingsvezel op de ontwikkeling van het metabool syndroom kan verklaren. Vervolgens hebben we de bijdrage van de vetcellen alsmede de macrofagen in het vetweefsel onderzocht betreffende het anti-inflammatoir effect van propionzuur. O.a. met behulp van kwantitatieve proteomics is gevonden dat de afgifte van ontstekingseiwitten door de macrofagen door propionzuur wordt geremd. Echter, geheel tegen onze verwachting in vonden

we dat ook de vetcellen zelf een rol konden spelen in de ontsteking door het uitscheiden van diverse ontstekingsstoffen en deze vetcellen konden ook T-cellen rekruteren hetgeen wijst op een belangrijke rol in ontstekingsprocessen. Tenslotte hebben wij de rol van de G-proteïne gekoppelde receptoren 41 en 43 alsmede Gi-o proteïne in het effect van propionzuur op vetweefsel onderzocht. Het effect op de meeste ontstekingsfactoren is Gi-o afhankelijk, hetgeen door een specifieke remmer kon worden aangetoond.

De resultaten in dit proefschrift werpen licht op het begrijpen van de interactie tussen dikke darm en vetweefsel fysiologie. Onze gegevens suggereren tevens dat het variëren van de propionzuur flux een mogelijke toepassing heeft in het onderdrukken van lichte en chronische ontsteking en daardoor beschermend werkt tegen metabool syndroom, diabetes type 2 en hart- en vaatziektes.

Propionate: a Candidate Metabolite to Link Colonic Metabolism to Human Adipose Tissue Inflammation

Acknowledgment

Acknowledgment

Finally this thesis has come to an end and it is the time to write a few last and personal words. While I am writing this section, the memories flashed back to me and it is actually beneficial in terms of judging myself and determining what went wrong and what went right and what I learned from it. Throughout my PhD I met many people, however, some of them had a strong influence on my life and I will never forget their names.

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Curriculum Vitae

Sa'ad Al-Lahham was born in Jerusalem-Palestine on 15th of January 1980. After he finished his high school in 1998, he started to study medicine in the Kharkov State Medical University-Ukraine, because his ambition was to prevent or treat diseases. Although he received an honor degree after the first year, he found that medical school is not the right place to gain the knowledge and tools that are necessary to discover solutions for the current serious diseases. Therefore, he left to Jordan to obtain his bachelor with an honor degree in Genetic Engineering and Biotechnology in the Jordan University of Science and Technology. Immediately after graduation, he worked for one year (2003-2004) as a teacher and research assistant in the Department of Biology and Biotechnology, Arab American University-Palestine. In the weekends he worked in Al-Quds University-Palestine on a research project that resulted in one research article. This project was about determining the genetic diversity of wheat landraces in Palestine and was funded by the United Nations Development Programme. In 2006, he obtained his Master degree in "Medical and Pharmaceutical Drug Innovation" at the University of Groningen-the Netherlands. A research article was published as his Master thesis. Immediately after he finished his master, he was offered a PhD position in a project from the Top Institute of Food and Nutrition and he conducted his research in the Medical Biomics Centre, University Medical Centre Groningen-the Netherlands. Now, he is still excited about his aim and consequently he will look for a new research job in the field of medical sciences.