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Nutritional Effects on Angiotensin-Converting Enzyme (ACE) and Its Determination by Genotype

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
HFD	High fat diet
LFD	Low fat diet
NUGAT	NUtriGenomic Analysis in Twins study
ASC	Human adipose stromal cells
PA	Pamitic acid
AA	Arachidonic acid
NF-κB	Nuclear factor-κB
20-HETE	20-hydroxyeicosatetraenoic acid
CVD	Cardiovascular disease
RAS	Renin-angiotensin system
AGT	Angiotensinogen
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
OGTT	Oral glucose tolerance test
REE	Resting energy expenditure
CID	Clinical investigation days
SNP	Single nucleotide polymorphism

Summary of publication-based thesis

1. Title

Nutritional Effects on Angiotensin-Converting Enzyme (ACE) and Its Determination by Genotype

2. Author

Xu Li

3. Abstract

Angiotensin-converting enzyme (ACE) is a critical component of the renin–angiotensin system (RAS) that not only regulates blood pressure and maintains the homeostasis of the cardiovascular system, but also has regulatory effects on lipid metabolism. In our NUtriGenomic Analysis in Twins (NUGAT) study, 46 pairs of healthy, nonobese twins were given a low-fat diet (LF, 55% carbohydrates, 30% fat, 15% protein) for 6 weeks to standardize for nutritional behavior, followed by a high-fat (HF, 40% carbohydrates, 45% fat, 15% protein) diet for 6 weeks under isocaloric conditions. After 6 weeks of HF diet, the expression level of ACE gene significantly increased both in circulation and in adipose tissue. We sought to examine the interaction of a frequent ACE genetic polymorphism with the nutritionally altered expression of the ACE gene. We observed that the presence of the ACE rs4343 gene polymorphism makes individuals of different genotypes have different susceptibility to the risk of hypertension and type 2 diabetes caused by HF diet. Specifically, HF diet had more adverse effects on circulating ACE levels, blood pressure and glucose metabolism in homozygotic twins with GG genotype. The interaction was confirmed in the MeSyBePo (Metabolic Syndrome Berlin Postdam) study. To investigate how HF diet increased the expression of ACE gene in adipose tissue, we conducted studies using human adipose stromal cells (ASCs) that were isolated from four patients undergoing bariatric surgery. Palmitic acid (PA) was added to the culture medium as a representative of saturated fatty acids, but it did not lead to significant changes in ACE mRNA expression and ACE activity compared to the fatty acid-free group. In contrast, arachidonic acid (AA), a representative of unsaturated fatty acids, decreased the ACE level in a dose-dependent manner, but the effect of 100 μM AA was reversed by 5 μM nuclear factor-κB (NF-κB) inhibitor BAY117082. Moreover, the presence of 10 μM of HET0016 – a specific inhibitor of the AA metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) - in the medium blocked the effect of AA on reducing ACE mRNA expression and ACE activity.

In summary, our data suggested that ACE is a potential molecular link between dietary fat intake and hypertension as well as cardiovascular disease. In addition, the interaction between the ACE rs4343 variant and dietary fat intake may influence the risk of hypertension and type 2 diabetes. The GG genotype represents a nutrigenetic marker for an adverse response to HF diet. In subcutaneous adipocytes cultured from morbidly obese individuals, AA can reduce ACE mRNA expression and ACE activity through NF-κB-dependent pathways.

4. ZUSAMMENFASSUNG

Das Angiotensin-Converting-Enzym (ACE) ist eine wichtige Komponente des Renin-Angiotensin-Systems (RAS), das den Blutdruck reguliert und die Homöostase des Herz-Kreislauf-Systems aufrechterhält. Es hat auch metabolische Steuerungseinflüsse. Studien haben gezeigt, dass sich der zirkulierende ACE-Spiegel bei Adipositas und Gewichtsverlust ändert. In unserer NUGAT-Studie (NUtriGenomic Analysis in Twins) haben wir gezeigt, dass eine Ernährung mit hohem Gehalt an gesättigten Fettsäuren (HF) den ACE-Spiegel erhöhen kann. In dieser Studie erhielten 46 Paare gesunder, nicht adipöser homozygoter und heterozygoter Zwillinge 6 Wochen lang eine fettarme (LF, 55% Kohlenhydrate, 30% Fett, 15% Eiweiß) Diät, die reich an Kohlenhydraten war, gefolgt von einer fettreichen (HF, 40% Kohlenhydrate, 45% Fett, 15% Eiweiß) Diät für 6 Wochen unter isokalorischen Bedingungen. Nach 6 Wochen HF-Diät erhöhte sich die Expression des ACE-Gens im Fettgewebe und die zirkulierenden ACE-Spiegel im Blut. Die Analyse des ACE-rs4343-Genpolymorphismus ergab, dass bei homozygoten Zwillingen des GG-Genotyps eine fettreiche Ernährung den zirkulierenden ACE-Spiegel erhöhte, ebenso wie den Blutdruck und die Glukosespiegel. Um den Mechanismus der HF-Diät zur Erhöhung der Expression des ACE-Gens im Fettgewebe weiter zu untersuchen, führten wir in-vitro-Studien mit humanen subkutanen Adipozyten durch. Dafür wurde subkutanes Gewebe von vier übergewichtigen Patienten im Rahmen einer bariatrischen Operation entnommen. Aus humanem subkutanem Fettgewebe wurden dann humane mesenchymale Stammzellen (MSC) isoliert und zu reifen Adipozyten in vitro differenziert. Die ACE-mRNA-Expression und die ACE-Aktivität in differenzierten Adipozyten wurden in Gegenwart verschiedener Stimuli gemessen. Nach einer Stimulation mit Palmitinsäure als Vertreter gesättigter Fettsäuren, gab es keine statistisch signifikante Änderungen der ACE-mRNA-Expression und der ACE-Aktivität. Die Stimulation mit Arachidonsäure (AA), als Vertreter ungesättigter Fettsäuren in verschiedenen Konzentrationen, führte zu einer dosisabhängigen Abnahme der ACE-mRNA Expression und der ACE-Aktivität in Adipozyten .

Diese Effekte bei 100 µM AA konnten durch eine Zugabe von 5 µM Nuclear factor-kappa B (NF-κB)-Inhibitor aufgehoben werden. Außerdem führte eine Zugabe von 10 µM 20-Hydroxyeicosatetraensäure (20-HETE)-spezifischem Inhibitor HET0016 zur Verringerung der ACE-mRNA-Expression und der ACE-Aktivität. Zusammenfassend kann eine fettreiche Ernährung die Expression und Aktivität von ACE im menschlichen Körper erhöhen, was darauf hindeutet, dass ACE ein potenzielles molekulares Bindeglied zwischen der fettreichen Ernährung und dem Bluthochdruck sowie den Herz-Kreislauf-Erkrankungen (CVD) darstellen kann. Die Existenz des ACE rs4343-Genpolymorphismus könnte dazu führen, dass Personen mit unterschiedlichen Genotypen unterschiedlich anfällig für das Risiko von Bluthochdruck, Herz-Kreislauf-Erkrankungen und Diabetes sind, die durch eine fettreiche Ernährung mit hohem Anteil gesättigter Fettsäuren verursacht werden.
In humanen differenzierten Adipozyten kann die AA, als eine ungesättigte Fettsäure, die ACE-mRNA-Expression und die ACE-Aktivität über den NF-κB-abhängigen Signalweg hemmen.

5. INTRODUCTION

The obesity epidemic continues to develop worldwide. According to the World Health Organization, the number of obese people in the world has nearly tripled since 1975. In 2016, more than 1.9 billion adults aged 18 years and above were overweight (body mass index, BMI ≥ 25), accounting for 39% of the total population; and more than 650 millions of them were obese (BMI ≥ 30), accounting for 13% of the total population[1]. Excessive central distribution of obesity, especially visceral fat, is associated with changes in hormone, inflammation, and metabolite levels. These changes stimulate some other mechanisms that contribute to hypertension status. It is estimated that obesity accounts for 65%–78% of essential hypertension cases[2, 3]. Obesity and high blood pressure often co-occur in the same patient. About 75% of hypertensive patients in Germany are overweight or obese[4]. A high-fat (HF) diet is well known as one of the most important factors associated with the development of obesity. High blood pressure caused by obesity has become a public health concern. However, the mechanisms of HF diet and increased blood pressure are complex and still insufficiently understood.
Angiotensin-converting enzyme (ACE), also known as peptidase P, angiotensin I-converting enzyme, CD143, and EC 3.4.15.1, is a membrane-bound protein, widely distributed in many tissues and cell types. Although it is mostly located in endothelial and epithelial cells, it has also been found in adipose tissue and adipocytes with other

renin–angiotensin system (RAS) components[5]. Approximately 30% of circulating angiotensinogen (AGT) is produced by adipose tissue[6]. Circulating ACE has been shown to decrease significantly during weight loss[7]. ACE converts the inactive angiotensin I to the active vasoconstrictor angiotensin II, which is involved in the control of blood pressure. Angiotensin-converting enzyme inhibitors (ACEIs) are widely used as a first-line drug for the treatment of hypertension worldwide[8].

The RAS is a classical regulatory system that is known for its role in blood pressure regulation and balance of fluid and electrolytes. It was originally described as a circulating hormonal system and a main cardiovascular regulator. The RAS is now considered a "pervasive" system, considering that it is expressed locally in various organs[9]. It is mainly expressed in renal tissues, cardiovascular system, adrenal glands, and liver, but it is also found in the brain, pancreas, adipose tissue, and some other tissues[10]. Local RASs located in different tissues are involved in physiological and pathophysiological processes independently from the systemic RAS through paracrine and autocrine modes, but they may still interact with the systemic RAS to play an endocrine role[11]. The systemic RAS overactivation is associated with the pathogenesis of obesity-related hypertension. In this system, AGT undergoes enzymatic cleavage by renin to produce a decapeptide angiotensin-I (1–10) (Ang-I). Ang-I is further converted to the vasoactive angiotensin II (1–8) (Ang-II) by ACE. ACE acts as an important vasoactive enzyme in the RAS, in addition to producing Ang-II, ACE degrades bradykinin by c-terminal cleavage of a dipeptide[12]. The most efficient Ang-(1-7)-generating enzyme is ACE2, which is known to be capable of generating Ang-(1–7) directly from Ang-II. In addition to this, Ang-(1-7) also can be formed indirectly from Ang-I through Ang-(1-9) intermediate, which is produced by Ang-I under the action of ACE2, and then cleaved by ACE to form Ang-(1-7). Nevertheless, the former pathway is more potent, since ACE2 has a 400-fold higher affinity for Ang-II than Ang-I[13]. In human endothelial cells, the expression of ACE is increased through the nuclear factor-kappaB (NF-κB) pathway[14]. Many genes involved in cardiovascular diseases (CVDs) are regulated by NF-κB. The *in-vitro* experiments showed that the activation of NF-κB was attenuated by ACE inhibitors in damaged vessels[15].

Moreover, several polymorphisms of ACE have been reported to express an association with blood pressure and adiposity[16]. The ACE gene contains a series of frequent polymorphisms that are in strong linkage disequilibrium with each other[17]. A frequent insertion/deletion (I/D) polymorphism located in the 16th intron of the ACE gene is apparently associated with an increased risk of cardiovascular diseases and type 2 diabetes[18, 19]. The ACE rs4343 is a silent non-coding single nucleotide

polymorphism (SNP) expressed at the mRNA level and serves as a proxy for the I/D polymorphism[20]. We also sought to find the genetic factors that influence ACE expression.

6. MATERIAL AND METHODS

6.1 NUtriGenomic Analysis in Twins (NUGAT) Study Design

The protocol was approved by the independent Ethical Review Board of the University of Charité-Universitätsmedizin, Berlin. The study was registered with ClinicalTrials.gov (Unique identifier: NCT01631123). In total, 46 pairs of healthy, nonobese twins were included in the study. There were 58 females and 34 males, and the age ranged from 18 to 70 years. The mean BMI was 22.8 ± 2.7 kg/m^2. Screening visits included physical examination, measurement of blood lipids and blood pressure, and oral glucose tolerance test (OGTT). Dietary interventions were designed under isocaloric conditions. The energy requirements of each participant were calculated based on the individual resting energy expenditure (REE). All participants followed a high-carbohydrate, low-fat diet (LF, 55% carbohydrates, 30% fat, 15% protein) for 6 weeks to standardize the nutritional behavior. Subsequently, they followed low-carbohydrate, high-fat diet (HF, 40% carbohydrates, 45% fat, 15% protein) for the following 6 weeks. The 55% carbohydrate, 30% fat and 15% protein is the standard diet model. However, in our experiment, we compared it with another diet model of 40% carbohydrate, 45% fat and 15% protein. LF and HF are labels used to distinguish the two diet models in this study[21]. Three clinical investigation days (CIDs) were organized, CID1 after 6 weeks of LF diet, CID2 after 1 week of HF diet, and CID3 after 6 weeks of HF diet. At each CID, anthropometric measurements, blood pressure, and blood analysis were conducted. To ensure participants' compliance, nutritionists offered periodic and specific dietary guidance throughout the intervention process. A majority amount of food was supplied to regulate participants' dietary behaviors[22].

6.2 ACE Genotyping

In the NUGAT study, genomic DNA was isolated using a commercial kit (NucleoSpin, Macherey-Nagel, Düren, Germany) from buffy coat samples and genotyped on HumanOmniExpressExome BeadChips (Illumina, Inc., San Diego, CA, USA). To confirm the results, samples from the Metabolic Syndrome Berlin Potsdam (MeSyBePo) study were used, which included 2385 Caucasian participants, among which 1992 participants were non-diabetic. Predesigned rs4343 TaqMan SNP Genotyping Assay (ViiA7 System; Applied Biosystems, Foster City, CA, USA) was used for genotyping[21, 22].

6.3 Isolation and differentiation of human adipose stromal cells

Human adipose stromal cells (ASCs) were isolated from adipose tissues of four patients participating in the LEMBAS (diet-induced changes in Liver fat and Energy Metabolism before Bariatric Surgery) study, which was registered at www.drks.de (DRKS00009509). All participants were obese patients with BMI > 40 kg/m² or BMI > 35 kg/m² and obesity-related comorbidities. The adipose tissue specimens used in our study were collected from four patients during Roux-en-Y gastric bypass surgery. The isolation protocol of ASCs adopted the improved method of Lee et al[23]. Briefly, large chunks of adipose tissues were first minced into approximately 5mg adipose tissues using sharp scissors. The minced tissues were poured through a 250μm funnel-shaped mesh and transferred into 50mL tubes filled with DMEM/F12 containing collagenase I (1 mL/g). Subsequently, the adipose tissues incubated in a water bath with constant shaking (100 rpm) at 37°C for 2 hours when there were nearly no complete AT blocks. Next, the digested AT was then filtered through a funnel with a 250μm mesh, and the flow containing ASCs was captured into a 50mL tube. The resuspended cells were cultured in Preadipocyte Growth Medium (Promo Cell, Heidelberg, Germany). In order to differentiate the ASCs, the cells were cultured in a differentiation solution after achieving 80%–90% confluence[24].

6.4 Oil red O staining

Oil red O staining confirmed the differentiation of mature adipocytes. After culturing the cells in maintenance media for 14 days, the differentiated adipocytes were collected and fixed in 10% formaldehyde for 1 hour. Next, adipocytes were washed with PBS and 60% isopropanol for 5 minutes. Thereafter, the cells were incubated in Oil Red O and analyzed under an optical microscope at x200 magnification[24].

6.5 Free fatty acids stimulation

For fatty acid treatment, palmitic acid (PA) was prepared using a modified method[25]. Briefly, PA was dissolved in ethanol with fatty acid-free bovine serum albumin (BSA), so that the final PA solution contained 1% BSA, 500μM PA, and 1% ethanol[26]. Arachidonic acid (AA) was mixed with 1% fatty acid-free BSA to produce the working solutions of 50, 100, or 200μM. All working solution diluted with DMEM were prepared before experiments. Seven days after adipocyte differentiation, the cell culture medium was changed to DMEM with 1% BSA with no fatty acids, or with PA 500μM, AA 50, 100, or 200μM. Next, based on the experimental results and other research reports, 100μM AA was selected for further experiments[27, 28]. To investigate the inhibitory effects of the NF-κB pathway, 5μM NF-κB inhibitor BAY117082[29] was

added into the cell culture medium for 1 hour, and then DMEM with the supplemental 0.1% BSA and 100μM AA was added and incubated over 24 hours. In addition, 20-hydroxyeicosatetraenoic acid (20-HETE) is a hydroxylation product of AA that is catalyzed by enzymes of the cytochrome P450. HET0016, a specific inhibitor of 20-HETE, was used to investigate the effects of AA on ACE expression and activity[30]. AA, BAY117082, and HET0016 were purchased from Cayman Chemical (Ann Arbor, MI, USA)[24].

6.6 RNA extraction and quantitative RT-PCR

Total RNAs were extracted from the adipose tissues and cultured adipocytes using Nucleospin® RNA II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Subsequently, the high-capacity cDNA reverse-transcription kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) was used to reverse-transcribe RNA into cDNA in accordance with the instructions. cDNA was synthesized from 1μg of RNA of each sample. Finally, gene expression detection was performed in triplicate by qRT-PCR assays using the ABI ViiA™7 Real-time PCR system (Applied Biosystems by Life Technologies, Carlsbad, CA, USA)[24]. The ACE primer sequences are forward primer 5'-CAGAACACCACTATCAAGCGGA-3', reverse primer 5'-CACGCTGTAGGTGGTTTCCAT-3.' The samples were normalized to a ribosomal protein large P0 (RPLP0). Forward primer 5'-GCTTCCTGGAGGGTGTCC-3', reverse primer 5'-GGACTCGTTTGTACCCGTTG-3'.

6.7 ACE activity

The ACE activity was determined according to the protocol described by Sentandreu and Toldra[31]. Briefly, 50 μL of diluted samples and standard solutions which were in dose-response of 8 concentrations were added to a 96-well microplate. The enzyme reaction began by adding 200 μL of fluorescent substrate (Abz-Gly-Phe (NO2)-Pro (Bachem, Bubendorf, Switzerland, cat. no. M-1100) at working solution. The first fluorescence was measured when the reaction started, and the second measurement was performed after 30 minutes of incubating at 37°C. The plate was read using a Tecan Infinite M200 microplate reader at an excitation and emission wavelength of 365nm and 425nm, respectively. The ACE activity was analyzed by the fluorescence generated during the incubation time[24].

6.8 Statistical analysis

Data are shown in figures as mean ± SD, if there is no special instruction. A Student's

t-test was used for a statistical comparison between the two groups ($p < 0.05$). Statistical significances among multiple groups were analyzed by one-way ANOVA ($p < 0.05$). Statistical analysis was processed using SPSS 20.0 (IBM SPSS, Chicago, IL, USA).

7. RESULTS

7.1 Effects of HF Diet on ACE levels in human subcutaneous adipose tissue

Quantitative real-time PCR showed that ACE mRNA expression in the human adipose tissue increased significantly after 6 weeks of HF diet (Figure 1[22]).

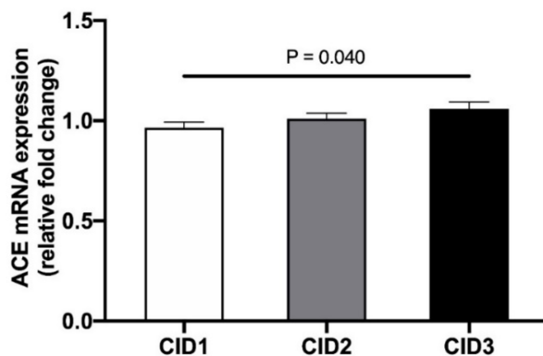

Figure 1. ACE mRNA expression in adipose tissue by qRT-PCR. Data are presented as mean ± SEM, *P* value is shown above. CID1, CID2 and CID3 indicate clinical investigation days after 6 weeks of LF diet, 1 week of the HF diet, and 6 weeks of the HF diet, respectively.

7.2 Validation of ACE rs4343 Genotype Effects on Blood Pressure and Type 2 Diabetes

In the NUGAT study, the distribution of genotype frequencies for ACE rs4343 polymorphism was shown as following: 31 homozygous carriers (AA), 44 heterozygous carriers (AG), and 17 variant homozygous carriers (GG). The ACE serum concentration was significantly different among different genotypes ($P < 0.001$). Specifically, AA showed the lowest, while GG had the highest ACE concentrations (Figure 2A[22]). Blood pressure levels among genotypes were not significantly different after 6 weeks of LF diet (AA/AG vs GG: 109 ± 13 mm Hg vs 115 ± 9 mm Hg, $P > 0.05$) or after 1 week of HF diet (AA/AG vs GG: 109 ± 12 mm Hg vs 113 ± 11 mm Hg, $P > 0.05$). The HF diet induced a significant increase in systolic blood pressure in twins with a GG-genotype only, while AA or AG-genotypes showed no change (AA/AG vs GG: 108 ± 12 mm Hg vs 117 ± 9 mm Hg, $P = 0.008$) (Figure 3A). In the MeSyBePo study, we confirmed that blood pressure had shown a significant correlation with ACE genotypes in individuals who had consumed diets with more

than 37% fat but not in those with a lower fat intake (AA/AG vs GG: 122 ± 16 mm Hg vs 131 ± 21 mm Hg, recessive model: $P_{SBP} = 0.008$, Figure 3B).
As for type 2 diabetes, the NUGAT study found a significant effect of the interaction of genotype and diet intervention on the fasting glucose concentrations (Figure 4a[21]). Fasting blood glucose levels in GG carriers increased by 0.5 ± 0.1 mmol/L (mean ± SEM), whereas no significant change was found in AA/AG carriers[21]. Changes in fasting blood glucose in response to HF diet also significantly differed between genotypes (Figure 4d[32]). A significant effect of the interaction between rs4343 genotype and the HF intervention was also observed on HOMA-IR. Namely, as shown in Figure 4c[32], HOMA-IR values increased in GG carriers with significantly higher concentrations at HF6 compared with AA/AG-carriers, and HOMA-IR measures did not change in AA/AG genotypes during HF diet.

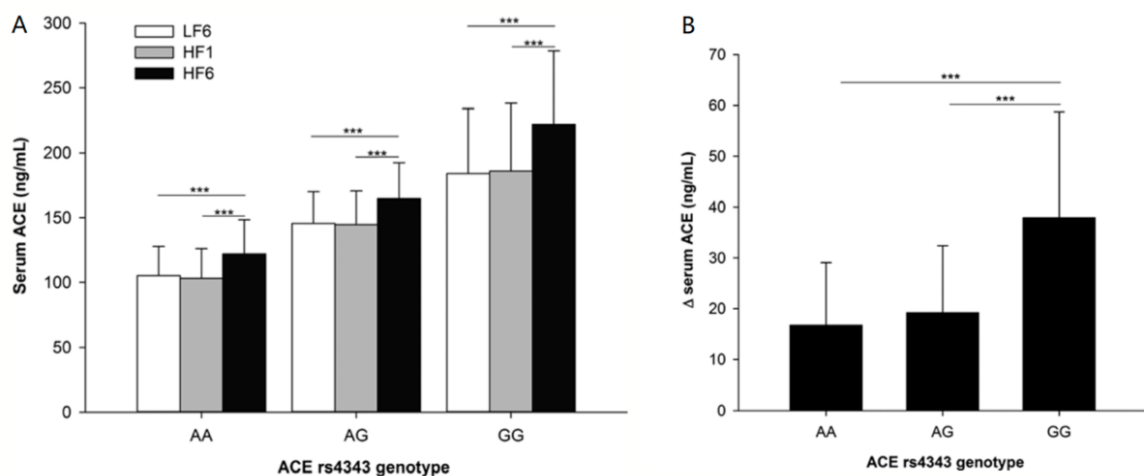

Figure 2. (A) Serum ACE levels in the NUGAT study stratified by ACE rs4343 genotypes and (B) the changes in serum ACE levels after 6 weeks of HF diet for different genotypes. All values are shown as mean ± SD of three experiments; $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$[22].

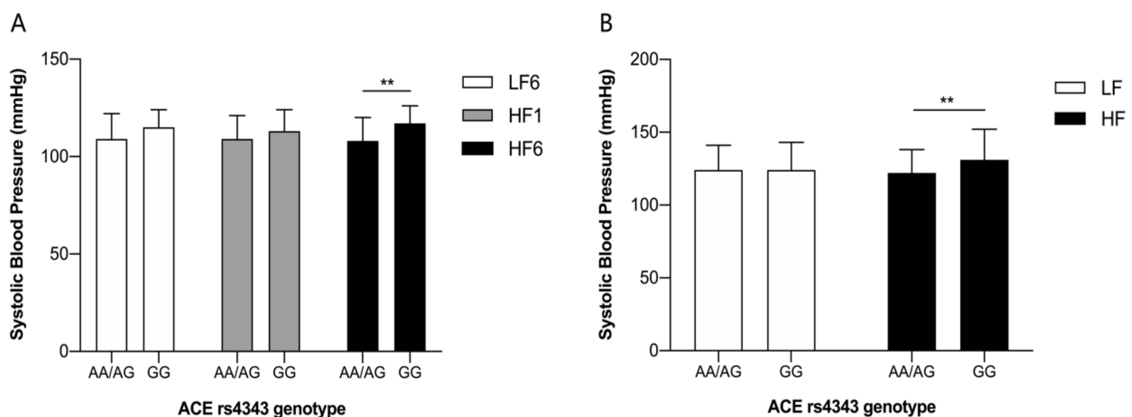

Figure 3. The effects of diet on systolic blood pressure stratified by ACE rs4343

genotypes in the NUGAT study (A) and Metabolic Syndrome Berlin Potsdam (MeSyBePo) study (B) (** $P < 0.01$). (A) In the NUGAT study, participants stratified by ACE rs4343 genotypes showed no differences in systolic blood pressure after 6-week LF diet (LF6) or subsequent 1-week HF diet (HF1). In contrast, 6-week HF diet (HF6) induced changes in blood pressure in GG carriers compared with AA/AG carriers. In the MeSyBePo study (B), GG carriers showed a higher systolic blood pressure after HF diet compared with AA/AG genotypes. All values are shown as mean ± SD in the above figures; *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

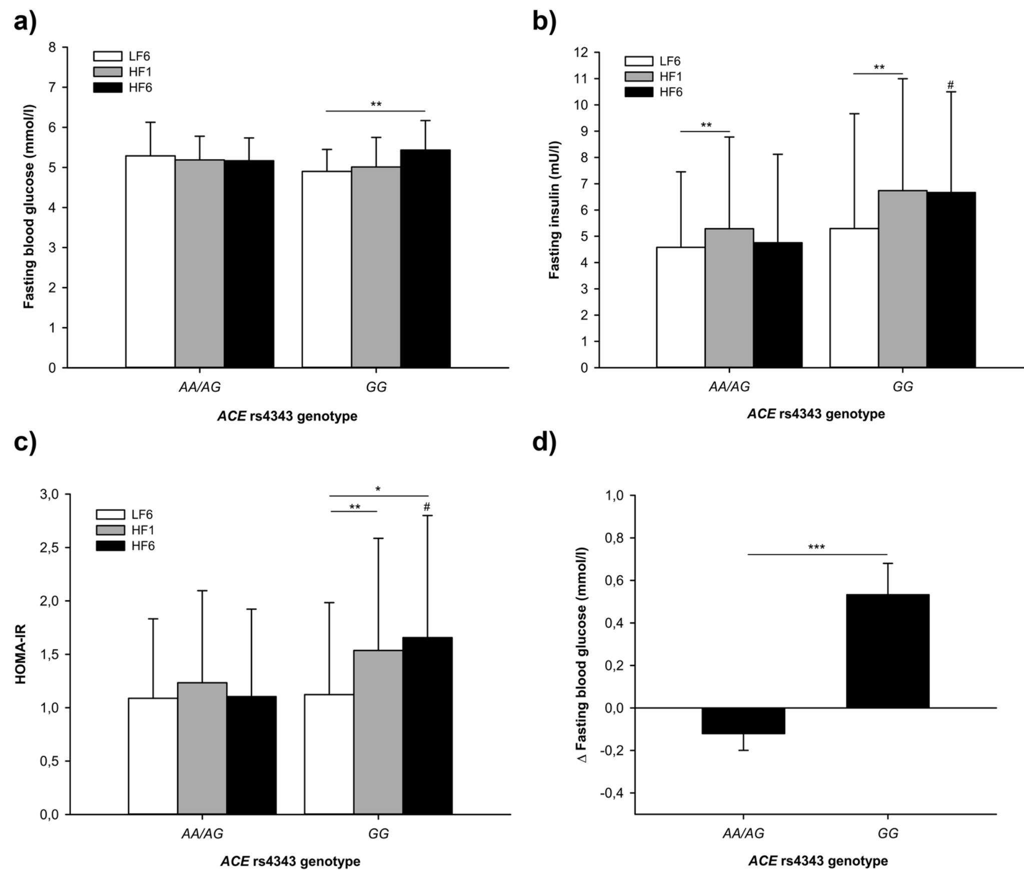

Figure 4. Parameters of glucose metabolism measured at time points LF6, HF1, and HF6 respectively or calculated, stratified by ACE rs4343 genotypes. (a) Fasting blood glucose, (b) Fasting insulin, (c) HOMA-IR, and (d) Δ Fasting blood glucose (HF6−LF6). All values are expressed as mean ± SD of three experiments; *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $^{\#}$ $P < 0.05$ vs AA/AG at HF6[21].

7.3 Evaluation of the cultured cells

Oil Red O staining in line with the previous protocol[23] was used to observe whether adipocyte differentiation was successful. The cytoplasm of cultured cells showed a typical red lipid droplet distribution (Figure 5F[24]).

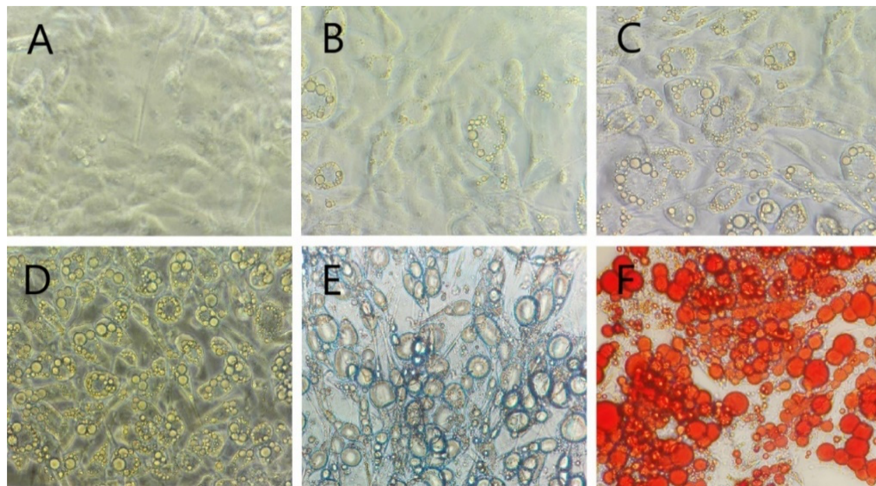

Figure 5. Microscopic images of differentiated human adipose stromal cells at various stages. Photographs were taken at day 1 (A), day 3 (B), day 7 (C), day 12 (D), day 14 (E) during differentiation, and after Oil Red O staining of lipid droplets (F).

7.4 Effects of different fatty acids on ACE expression in adipocytes

PA, a representative of saturated fatty acids, was used to treat the adipocytes. PA did not affect the mRNA expression and activity of ACE (Figure 6a, b[24]). However, AA, an unsaturated fatty acid, decreased the mRNA expression and activity of ACE in a dose-dependent manner (Figure 6c, d[24]). AA inhibited ACE gene expression in adipocytes at the dose of 100μM, thereby reducing the activity of ACE in the culture medium (Figure 6e, f[24]). For further experiment of the AA effect, the 100μM concentration was selected[28].

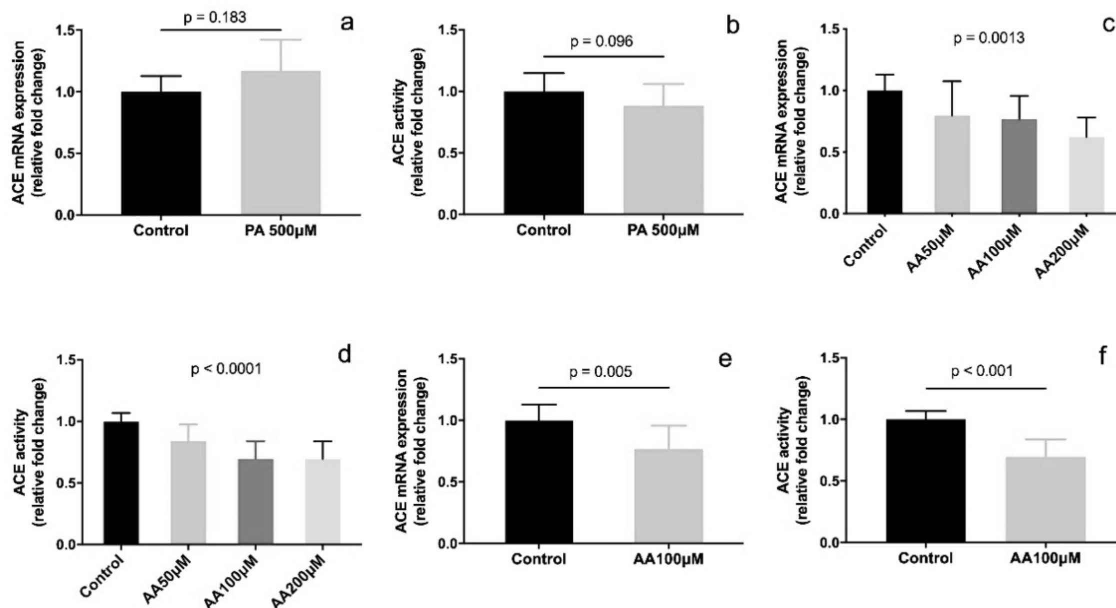

Figure 6. Effects of palmitic acid (PA) and arachidonic acid (AA) on mRNA level and activity of angiotensin-converting enzyme (ACE). (a) ACE mRNA expression in the presence of PA. (b) ACE activity in the culture medium under 500μM of PA. (c, d) The expression of ACE mRNA (c) and ACE activity (d) treated with different concentrations of AA (50μM, 100μM, 200μM) significantly decreased with increasing concentration of AA (n = 3). (e, f) The effect of 100μM AA on ACE mRNA expression (e) and ACE activity (f). All values are expressed as mean ± SD of three replicates[24].

7.5 Involvement of the NF-κB pathway in the inhibition of ACE in adipocytes

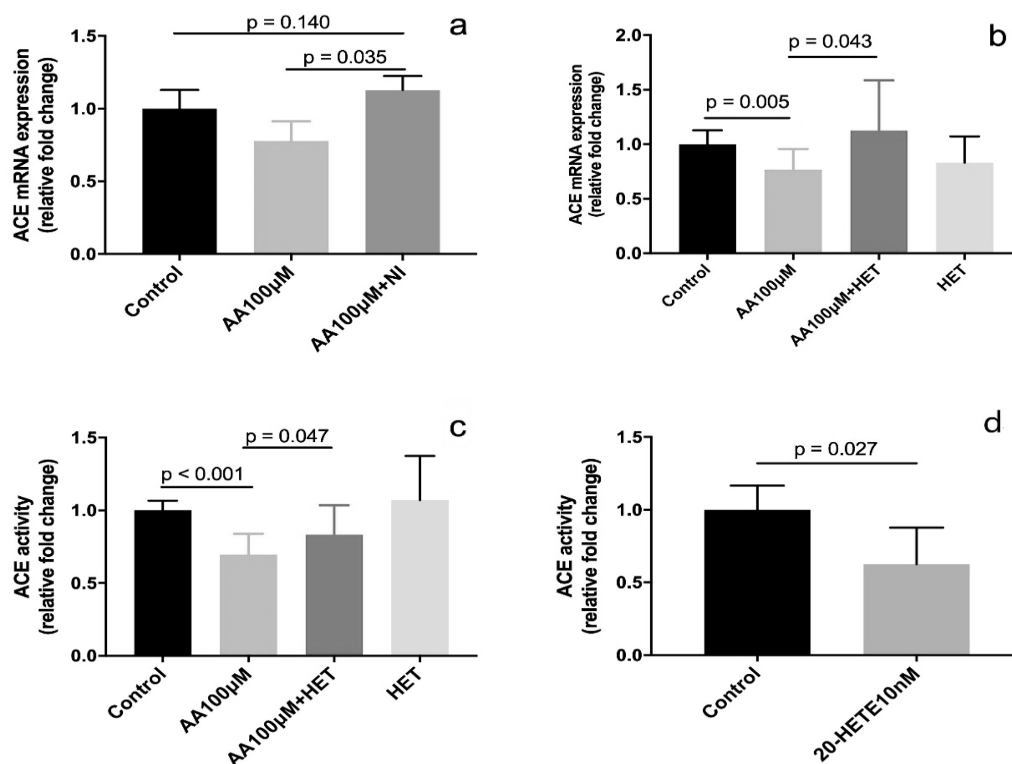

Figure 7. Effects of NF-κB inhibitor BAY117082 (NI) and 20-HETE inhibitor HET0016 (HET) on ACE mRNA expression and activity in the presence of 100μM AA. (a) 5μM NI reversed the inhibitory effect of 100μM AA on ACE mRNA expression (n = 3). (b, c) Reversal of the AA-induced inhibition of ACE mRNA expression (b) and ACE activity (c) by 10μM HET0016 (HET) (n = 3). (d) Finally, 10nM 20-HETE decreased ACE activity in the cell culture media. All values are given as mean ± SD of three experiments[24].

Free fatty acids have been reported to activate the RAS in adipose tissue through the NF-κB pathway[29]. Based on this, we investigated whether ACE expression changes when NF-κB pathway is inhibited. NF-κB pathway inhibitor BAY117082 was used to treat the cultured adipocytes in the presence of AA. Compared with the control cells,

BAY117082 increased ACE expression significantly (Figure 7a[24]). 20-HETE is a metabolite of the cytochrome P450 (CYP) pathway of AA and plays a complex role in blood pressure regulation[33,34]. We found that the addition of 20-HETE inhibitor HET0016 affected the effect of AA. HET00116 could rescue the effect of AA on ACE gene expression and activity (Figure 7b, c[24]), but the ACE activity was inhibited when 10nM 20-HETE was added to the adipocyte culture medium (Figure 7d[24]). The result indicated that inhibition of NF-κB pathway is related to 20-HETE metabolism.

8. DISCUSSION

In the NUGAT study, we found an increase in circulating levels of ACE and adipose tissue ACE in 92 normal-weight healthy participants after 6 weeks of HF diet with 45% total fat and 18% saturated fat. In the 6 weeks before the HF diet, the twins had consumed LF diet with 30% total fat and 10% saturated fat. Since ACE works by converting Ang-I to the vasoconstrictor Ang-II, it is inferred from the study that HF diet may increase the risk of hypertension by increasing ACE gene expression levels, considering that ACE is a well-known parameter linked to CVD.

Moreover, we investigated the effects of different types of fatty acids on ACE gene expression. PA (C16:0) is most abundant saturated fatty acid (SFA) in human tissues. The mean plasma PA concentration in a young healthy human body is 1631μM[32] . High intake of PA has been linked with adverse cardiovascular events[33-35]. In our study, PA stimulation appeared to have no effect on ACE gene expression in *in-vitro* (Figure 6a[24]). These data suggested that the increase in ACE in a HF diet was not a direct result of PA interaction with adipocytes, which is likely because PA may be stored after esterification in human adipose tissue or is detoxified by desaturation to palmitoleic acid.

AA is the main component of membrane lipids and belongs to the n-6 polyunsaturated fatty acids (PUFA). It is obtained from food or through the gradual desaturation and chain elongation of the essential fatty acid linoleic acid (LA)[36]. Linoleic acid is a predominant n-6 fatty acid in some plant oils. However, increased intake of linoleic acid does not necessarily result in increased levels of AA in humans[37]. AA is widely believed to increase inflammation because it is a precursor to many powerful pro-inflammatory mediators, including prostaglandins and leukotrienes. Nonetheless, a study in human showed that increased intake of AA supplementation did not significantly alter the concentrations of inflammatory mediator e.g., PGE2 or LXA4[38]. A previous meta-analysis showed that higher levels of AA consumption significantly reduced risk of heart disease and had positive effect on CVD outcomes[39].

In this assay, we selected AA as an example of unsaturated fatty acids to stimulate adipocytes. Our study found that in the presence of AA, the expression of ACE in the cultured adipocytes decreased (Figure 6e[24]) and activity of ACE in the nutrient medium decreased (Figure 6f[24]), which may indicate a protective effect. AA was reported to stimulate preadipocyte differentiation in a cyclooxygenase-dependent manner[40]. Our results are consistent with the previous study[28].

Because of albumin binding and transport to cells, circulating concentrations of free AA are usually very low[41]. Plasma concentrations of total AA range from 42.7μM to 882.8μM[32]. Interestingly, 50μM to 150μM free AA exhibits cytotoxicity in *in-vitro* experiments, which is why the plasma AA concentration is likely to be much lower due to binding to albumin and other proteins in humans[42]. The total free fatty acids to BSA serum ratio is no more than 3.1:1 in healthy body[43]. In our experiments, 1% (w/v) BSA was used, and the highest molar ratio of AA to BSA was 1.3:1, which is in line with the molar ratio under physiological conditions. And AA has higher affinity for albumin than other fatty acids[44]. Free AA is processed by four enzyme pathways to produce highly bioactive metabolites that participate in a variety of important processes. These pathways include cyclooxygenase (COX), lipoxygenase (LOX), cytochrome p450, and anandamide. The CYP 450 pathway contains two vital enzymes: CYP450 epoxygenase, and CYP450 ω-hydroxylase, which produce EETs and 20-HETE, respectively. Free AA can also be metabolized by nonenzymic reactions. Four double bonds of AA are easily oxidized to form bioactive molecules[41]. In addition, 20-HETE levels are increased by Ang-II while 20-HETE inhibitors attenuate the vasoconstrictor and hypertensive response to Ang-II[45, 46].

Moreover, 20-HETE increases the size and proliferation of fat cells[47], and its adverse effects include oxidative stress and increasing BMI, so it is related to metabolic syndrome. Evidence suggests that there is a low level of endogenous 20-HETE in adipocytes, i.e., during the differentiation process of adipocytes derived from human mesenchymal stem cells, the expression level of the main 20-HETE synthase CYP4F2 is reduced. At the same time, the exogenous introduction of 20-HETE (0.1–1μM) increases lipogenesis in a dose-dependent manner[47]. Previous studies confirmed that 20-HETE can activate NF-κB[48]. Our study found that the addition of HET0016, a selective 20-HETE synthesis inhibitor, reversed both the inhibition of ACE expression and ACE activity by AA (Figure 7b, c[24]). Moreover, when merely 10nM of 20-HETE was added into the medium, the ACE activity of unpretreated adipocytes was downregulated (Figure 7d[24]). Therefore, we hypothesized that the effect of AA on inhibiting ACE transcription and ACE activity may be partially exerted by its metabolite 20-HETE and that inhibiting 20-HETE synthesis could reverse the ACE

inhibition effect. In previous reports, 20-HETE activated the NF-κB pathway and promoted the expression of ACE[49]. Therefore, we also introduced an inhibitor of NF-κB in this study. As a result, inhibition of the NF-κB pathway reversed the AA-induced reduction in ACE expression (Figure 7a[24]). Our findings are different from the previous report[49], which may be mainly due to differences in tissue and cell types. Specifically, our cell culture used human primary adipocytes. Although there is no difference between preadipocytes in DM and nondiabetic (NDM) patients in accumulating cytoplasmic lipids and upregulating the expression of adipogenic genes[50], it has been found that different types of fatty acids in the diet have different effects on the differentiation and proliferation of preadipocytes[51]. It is undeniable that patients may experience epigenetic changes under different metabolic states. In addition, we believe that the effect of fatty acids on ACE may be more significant in the adipocytes of obese patients and type 2 diabetes patients.

Previous reports usually used adipocytes derived from mouse cell lines 3T3-L1[29], but mouse cell lines had limitations[23]. Other studies used human endothelial cells or adipocytes but from other tissues. To my knowledge, this is the first time to use mature adipocytes derived from stromal cells extracted from human subcutaneous adipose tissue to evaluate the ACE-related effects of AA. In immunohistochemistry, negative markers such as CD31, CD34 or CD45 are used to define the stromal cells derived from tissue specimens. However, this is debatable. For example, quite a few ASCs are CD34 positive[52]. Although we did not measure the markers, the method we used to differentiate and culture adipocytes was strictly implemented in accordance with the cited literature[23], and the medium (Preadipocyte Growth Medium) we used in the experiment was a special medium only for adipocyte growth. The ASCs we used in our study were plastic adherent and had the capacity of differentiation into adipocytes[53]. We confirmed the adipocyte by Oil red O staining. Figure 5F showed a typical red lipid droplet distribution. Furthermore, the method may also be found in other studies from our department[54]. Nevertheless, the ASCs used in this study was isolated from four morbidly obese patients by surgical biopsy, and their metabolic status may have induced epigenetic changes, which may have led to abnormal cell metabolism. The ASCs may also differ in differentiation and physiological function from adipocytes in nonobese humans[23].

Moreover, the RAS regulation varies among adipose cells in different tissues. For example, it has been reported that a HF diet can increase the expression of Ang-II in visceral fat without affecting the expression of Ang-II genes in subcutaneous white fat and brown adipose tissue[55]. Therefore, the results of this study indicate that different

cell types are crucial to the study of RAS function and the expression of its components. However, additional animal or human studies are needed to confirm this conclusion.

In addition, the ACE gene contains A series of frequent polymorphisms, in which the ACE rs4343 variant is in a nearly perfect linkage imbalance with the ACE I/D polymorphism, in which the A allele corresponds to the insertion (I) variant and the G allele corresponds to the deletion (D) variant. It can be used as a surrogate marker for I/D polymorphism. Many studies have shown associations of the ACE genotype, albeit inconsistently, with increased risk of CVD and metabolic disease[29,71,72]. Our results showed that the ACE level in the volunteers in NUGAT study carrying the homozygous GG was significantly higher than that of the AG and AA carriers (Figure 3[22]). The difference persisted when given a HF diet but only in the carriers of the GG genotype. GG carriers had an increased risk of hypertension compared with AG/AA carriers. The results were confirmed in a second cross sectional study, MeSyBePo. Our data also showed that the interaction between ACE rs4343 variant and dietary fat intake significantly affected glucose metabolism; specifically, homozygous carriers of the GG allele were more sensitive to negative reactions to HF diet and had an increased the risk of type 2 diabetes in healthy and nonobese subjects[21]. Therefore, ACE rs4343 gene polymorphism is a powerful nutritional marker that regulates the blood pressure elevation and the development of type 2 diabetes. Our results may explain the inconsistent association of the I/D-genotype of the ACE gene with cardiovascular disease and type 2 diabetes, and call for a reevaluation with reference to nutritional data were possible. Whether the impact of the ACE-variant would be blocked by ACE-inhibitors is currently unknown and represents a relevant question for further research. Limitations of our data in NUGAT apply to the relatively small cohorts, the inclusion of Caucasians only and lack of investigations in obese as compared to normal weight participants.

The in vitro studies might be extended by further analysis of other lipids and more detailed analysis of the mechanisms involved, including genetic knock down.

To summarize, we found that HF diet induced ACE gene expression in the adipose tissue, revealing ACE to be a possible biomarker associated with nutritional effects and CVD. Saturated fatty acids and unsaturated fatty acids had different effects on the production of ACE in human subcutaneous adipocytes. Preliminary research evidence of the related mechanism suggested that they may be intervention targets for RAS and related pathophysiological events in the metabolic pathway of adipocytes. Moreover, our results suggested an underlying gene–diet interaction. ACE rs4343 gene polymorphism is a vital nutrition-related chronic disease risk marker, which may be used for nutritional dietary guidance.

9. References

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Affidavit (Statement of contributions)

Statutory Declaration

"I, Xu L, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Nutritional Effects on Angiotensin Converting Enzyme (ACE) and Its Determination by Genotype" independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

[In the case of having conducted your doctoral research project completely or in part within a working group:] Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date____________________ Signature________________________

STATEMENT OF CONTRIBUTIONS

Xu L has contributed to the following publications as follows:

Publication 1: **Xu L**, Schüler R, Xu C, Seebeck N, Markova M, Murahovschi V, Pfeiffer AFH. Arachidonic acid inhibits the production of angiotensin-converting enzyme in human primary adipocytes via a NF-κB-dependent pathway. Ann Transl Med. 2020 Dec.

Detailed involvement: Xu L performed most of the experiments, acquisition of results, statistical analysis and interpretation of the data in this paper, including human stem cells culture, Oil Red O staining for the mature adipocytes confirmation, free fatty acids preparing, quantitative real-time PCR for the ACE mRNA expression and ACE enzyme activity measurement. Xu L illustrated all the figures and wrote the manuscript.

Publication 2: Schüler R, Osterhoff MA, Frahnow T, Seltmann AC, Busjahn A, Kabisch S, **Xu L**, Mosig AS, Spranger J, Möhlig M, Hornemann S, Kruse M, Pfeiffer AF. High-Saturated-Fat Diet Increases Circulating Angiotensin-Converting Enzyme, Which Is Enhanced by the rs4343 Polymorphism Defining Persons at Risk of Nutrient-Dependent Increases of Blood Pressure. J Am Heart Assoc. 2017 Jan.

Detailed involvement: Xu L performed the experiments including genomic DNA isolating and quantitative real-time PCR for ACE mRNA expression measurement and performed the statistical analysis, as shown in Figure 3 correspondingly. And Xu L critically reviewed the manuscript.

Publication 3: Schüler R, Osterhoff MA, Frahnow T, Möhlig M, Spranger J, Stefanovski D, Bergman RN, **Xu L,** Seltmann AC, Kabisch S, Hornemann S, Kruse M, Pfeiffer AFH. Dietary Fat Intake Modulates Effects of a Frequent ACE Gene Variant on Glucose Tolerance with association to Type 2 Diabetes. Sci Rep. 2017 Aug.

Detailed involvement: Xu L performed the genomic DNA isolating from buffy coat for genotyping, data acquisition and interpretation according to Figure 2-3. Xu L critically reviewed the manuscript.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

SELECTED PUBLICATIONS

Publication 1

1- **Xu L**, Schüler R, Xu C, Seebeck N, Markova M, Murahovschi V, Pfeiffer AFH. Arachidonic acid inhibits the production of angiotensin-converting enzyme in human primary adipocytes via a NF-κB-dependent pathway. Ann Transl Med. 2020 Dec;8(24):1652. doi: 10.21037/atm-20-7514. PMID: 33490164; PMCID: PMC7812212.
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Arachidonic acid inhibits the production of angiotensin-converting enzyme in human primary adipocytes via a NF-κB-dependent pathway

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Background: The modulating mechanism of fatty acids on angiotensin-converting enzyme production (ACE) in human adipocytes is still elusive. Diet-induced regulation of the renin angiotensin system is thought to be involved in obesity and hypertension, and several previous studies have used mouse cell lines such as 3T3-L1 to investigate this. This study was carried out in human subcutaneous adipocytes for better understanding of the mechanism.

Methods: Human adipose stem cells were isolated from subcutaneous adipose tissue biopsies collected from four patients during bariatric surgery and differentiated into mature adipocytes. The mRNA expression and the activity of ACE were measured under different stimuli in cell cultures.

Results: Arachidonic acid (AA) decreased ACE mRNA expression and ACE activity in a dose-dependent manner while palmitic acid had no effect. The decrease of ACE by 100 μM AA was reversed by the addition of 5 μM nuclear factor-κB (NF-κB) inhibitor. Furthermore, when the production of 20-hydroxyeicosatetraenoic acid, a metabolite of AA, was stopped by the specific inhibitor HET0016 (10 μM) in the culture media, the effect of AA was blocked.

Conclusions: This study indicated that AA can decrease the expression and activity of ACE in cultured human adipocytes, via an inflammatory NF-κB-dependent pathway. Blocking 20-hydroxyeicosatetraenoic acid attenuated the ACE-decreasing effects of AA.

Keywords: Fatty acids; human adipose stem cells; 20-hydroxyeicosatetraenoic acid

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Introduction

The World Health Organization defines hypertension as a pathological status in which "the blood vessels have persistently raised pressure". The deleterious consequence of hypertension is the damage it causes to affected organs, leading to an increased risk of nephropathy, vasculopathy, cardiovascular, or cerebrovascular events (1). Pathophysiologically, risk factors for hypertension include obesity, smoking, family history, high salt food intake and an overall unhealthy lifestyle and diet. Among the currently available medications for treating hypertension, angiotensin-converting enzyme (ACE) inhibitors and

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angiotensin receptor blockers are recommended as first choices (2).

ACE is a membrane-bound protein that indirectly increases blood pressure by converting angiotensin I to the active angiotensin II, a vasoconstrictor. ACE is most commonly found in endothelial and epithelial cells. ACE, in association with the other renin angiotensin system (RAS) components, was reported to be expressed in adipose tissue and cultured adipocytes (3). It is estimated that nearly 30% of the circulating angiotensin is produced by adipose tissue (4,5).

Obesity is a well-known risk factor for hypertension, and obesity-related hypertension has been ascribed to an over-activated RAS (6). The activity of the adipose tissue RAS contributes to systemic high blood pressure and chronic inflammation in adipose tissue (7,8). We recently reported that a high fat diet induced an increase in ACE expression in individuals with a genetic susceptibility and was associated with increased blood pressure and elevated blood glucose in a clinical human study (9,10).

Arachidonic acid (AA) is a long-chain omega-6 (n-6) polyunsaturated fatty acid (PUFA) which is obtained from food or by stepwise desaturation and chain elongation of linoleic acid (LA), an essential fatty acid (EFA) (11). Marine fish, animal tissues and eggs are the major supply of AA, algae and some plants were also reported as potential sources of AA (12-14). Because of the lack of biosynthesis enzymes, humans and other mammals cannot directly synthesize AA. Therefore, they have to obtain enough AA via food or dietary intake of its precursors (15). AA is the main component of membrane lipids, and mainly metabolized by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450). AA could be converted into various metabolites such as inflammatory lipids or eicosanoids (16). AA can be converted into prostaglandins (PGs) and thromboxanes (TXs) by the cyclooxygenase (COX) pathway, the metabolites of this pathway play an important role in vessel tone regulation, mediating platelet aggregation and immune response (17-19). Through the lipoxygenase (LOX) pathway, AA can be metabolized into leukotrienes (LTs) and lipoxins (LXs). Lipoxins mainly exhibit anti-inflammatory properties (20). Besides, epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs) are generated through the cytochrome P450 (CYP450) pathway. And these compounds play a main part in the modulating of kidney, lung, and cardiovascular function (21). 20-HETE is considered a potent vasoconstrictor by various means. However, it shows a potential conflicting role in regulating renal hypertension (22).

In human endothelial cells, angiotensin-converting enzyme (ACE) mRNA expression and ACE activity are increased via nuclear factor-κB (NF-κB) pathway (23), many genes involved in vascular physiopathology are regulated by NF-κB. NF-κB activation was diminished *in vivo* in injured vessels by angiotensin-converting enzyme inhibitors (24). However, the mechanism involved in modulating ACE expression in adipose tissue remains elusive, and this study therefore aimed to investigate the effects of fatty acids on ACE expression and activity in human adipocytes.

In this study, primary human adipocytes were differentiated and cultured in the presence of unsaturated and saturated fatty acids. The ACE expression in the cells and ACE activity in the culture media were measured. The potential relationship between ACE and fatty acids was assessed via artificial perturbation of the NF-κB inflammatory pathway.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7514).

Methods

Cell isolation and culture

Human adipose stem cells (ASCs) were isolated from four patients who were participants in the LEMBAS study (diet-induced changes in Liver fat and Energy Metabolism prior to Bariatric Surgery) (25). This clinical intervention study was approved by the Ethics Committee of the Charité University Medicine in Berlin (Application no.EA4/006/15) in accordance with the Declaration of Helsinki and registered at www.drks.de (DRKS00009509). All four study participants were morbidly obese with a body mass index (BMI) above 40 kg/m^2. Their subcutaneous adipose tissue biopsies were collected during the Roux-en-Y gastric bypass operation. Informed consent was acquired from each individual prior to surgery.

ASCs were isolated following the method modified from Lee *et al.* (26). Briefly, 5 mg (2–3 mm^3) of adipose tissue (AT) was minced into small pieces. The minced tissue was rinsed by phosphate buffer saline (PBS), passed through a 250 µm funnel-shaped mesh and then digested in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Thermo Fisher Scientific, Waltham, MA, USA) containing collagenase I (1 mg/mL) (Sigma Aldrich

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Chemie, Steinheim, Germany) at 37 °C for 2 hours with constant shaking (100 rpm). Subsequently, the digested AT was filtered through a 250 µm mesh and the filtrate, which contains the ASCs, was collected and centrifuged at 500 ×g for 10 minutes. Afterwards, the upper fat layer and the middle medium layer above the cell pellet in the tube were removed. The cells were resuspended after adding red blood cell (RBC) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) to diminish the RBC cells for better attachment. After centrifugation at 500 ×g for 5 minutes, the cells were resuspended again with Preadipocyte Growth Medium containing Preadipocyte Growth Medium SupplementMix (Promo Cell, Heidelberg, Germany) and 1% penicillin/streptomycin (Sigma Aldrich Chemie, Steinheim, Germany) and cultured in a 15 mL flask. Cells were maintained in a 37 °C incubator with 5% CO_2 and refed every 2–3 days until 80–90% confluence, and then cultured for 14 days in 12-well plates in differentiation medium which contained DMEM/F12, 1% penicillin/streptomycin, 500 µM IBMX, 25 nM dexamethasone, 0.2 nM triiodothyronine (T3), 33 µM D-biotin, 15 mM pantothenate, 20 nM human insulin, 0.01 mg/mL transferrin, and 2 µM rosiglitazone (all chemicals were purchased from Sigma Aldrich Chemie, Steinheim, Germany). Differentiation of the mature adipocytes was confirmed by oil red O stain. NF-κB inhibitor BAY117082 and 20-hydroxyeicosatetraenoic acid (20-HETE) production inhibitor HET0016 (27) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Oil red O staining

After 14 days of adipogenesis, differentiated adipocytes were washed with sterile PBS and fixed in 10% formaldehyde (Sigma Aldrich Chemie, Steinheim, Germany) for 1 hour of incubation at room temperature. Oil red O was prepared by mixing oil red O stock solution with deionized water in the ratio of 3:2. Thereafter, adipocytes were gently rinsed with water, and 60% isopropanol (Sigma Aldrich Chemie, Steinheim, Germany) was added for 5 minutes. Finally, the adipogenic cultures were incubated in oil red O for 10 minutes, rinsed with tap water until the water ran clear, and analyzed under the microscope.

Free fatty acids preparation and treatment

Palmitate (Sigma Aldrich Chemie, Steinheim, Germany) was dissolved completely in ethanol at 70 °C and then complexed with fatty acid-free bovine serum albumin (BSA) (Sigma Aldrich Chemie, Steinheim, Germany) at 55 °C for 10 minutes yielding the final palmitic acid (PA) stock solution of 5 mM. The working solution of 500 µM (28-30) was prepared with DMEM before experiments.

Arachidonic acid (AA) (Cayman Chemical, Ann Arbor, Mi, USA) was complexed with fatty acid-free BSA. The working solutions of 50, 100, or 200 µM were prepared with DMEM before the experiments.

After differentiation for 7 days (approximately 80% differentiation as estimated by lipid droplets), the cell culture medium was changed to DMEM with 1% BSA and without fatty acids, or with PA 500, AA 50, 100, or 200 µM, for the purpose of determining the effects of saturated and unsaturated fatty acids. Subsequently, AA 100 µM was used in further experiments (see results section) and other research reports (31-33). To explore the effects of NF-κB pathway inhibition, adipocytes were pretreated with 5 µM BAY117082 for 1 hour and then treated with either DMEM + 0.1% BSA or DMEM + 0.1% BSA + 100 µM AA for 24 hours. In order to ascertain the effects of 20-HETE, adipocytes were pretreated with 10 µM HET0016, the specific inhibitor of 20-HETE (27), for 1 hour and then treated with either DMEM + 0.1% BSA or DMEM + 0.1% BSA + 100 µM AA for 24 hours.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted by using the Nucleospin® RNA II Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. cDNA was synthesized from 1 µg of RNA of each sample by using the high capacity cDNA reverse transcription kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). QRT-PCR was performed using the power SYBR Green PCR master mix (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and detected in triplicates with the ABI ViiA™7 Real time PCR system (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). The samples were normalized to a ribosomal protein large P0 (RPLP0). The primers synthesized by Thermo Fisher Scientific (Waltham, MA, USA) are shown in *Table 1*.

Assessment of ACE activity

ACE activity was determined in triplicate by measuring the fluorescence of the product generated by the specific

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Table 1 Primers used in the study

Gene	Forward	Reverse
RPLP0	5'-GCTTCCTGGAGGGTGTCC-3'	5'-GGACTCGTTTGTACCCGTTG-3'
ACE	5'-CAGAACACCACTATCAAGCGGA-3'	5'-CACGCTGTAGGTGGTTTCCAT-3'

ACE, angiotensin-converting enzyme.

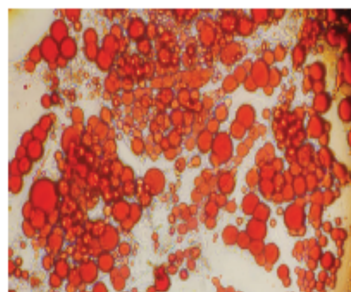

Figure 1 The oil red O staining of differentiated cells photographed using a microscope (×100). Lipid droplets are stained in red.

substrate Abz-Gly-Phe (NO_2)-Pro (Bachem, Bubendorf, Switzerland, cat. no. M-1100). The protocol designed by Sentandreu *et al.* was followed (34). Briefly, 50 µL testing samples and standard solutions of eight different concentrations were added into a 96-well microplate. Captopril-inhibition and blank-contrast wells were also included. A 200 µL substrate working solution, which was mixed by diluting M1100 in 150 mM Tris-base buffer (pH 8.3) with 1.125 M NaCl, was added into each well to initiate the enzyme reaction. Fifty µL of samples were incubated in a final volume of 300 µL with 0.45 mM of specific substrate. The plate was read two times with a Tecan infinite M200 microplate reader at an excitation wavelength of 365 nm, and an emission wavelength of 425 nm. The first measurement was performed at room temperature when the reaction was initiated, and the second after incubating the solution at 37 ℃ for 30 minutes. ACE activity was determined by the fluorescence emission differences between the incubation periods.

Statistical analysis

Statistical data were processed using SPSS 20.0 (IBM SPSS, Chicago, IL, USA). Results are expressed as the mean ± SD and data were analyzed using student's *t*-test for unpaired samples or the one-way ANOVA for multiple comparisons among groups.

Results

Evaluation of the cultured cells

In order to ascertain the success of the adipocyte differentiation, oil red O staining was performed. The cultured cells were characterized with typical red oil-stained lipid drops distributed in the cytosol (*Figure 1*). At least 80% of the cells were fat cells according to the staining. Combined with some morphological features, the cultured cells qualified for the current study according to the optimal protocol (26).

ACE expression in the presence of different fatty acids

Two kinds of fatty acids were selected to stimulate the adipocytes. For the saturated fatty acids, PA was selected as the example. Neither the mRNA expression of ACE nor the ACE activity in the culture media were affected by the addition of PA (*Figure 2A,B*). On the contrary, in the presence of AA, an inflammation-related unsaturated fatty acid, both the mRNA expression of ACE and the ACE activity in the culture media decreased dose-dependently (*Figure 2C,D*). At the dose of 100 µM, AA inhibited the expression of ACE in adipocytes, which in turn resulted in lowered ACE activity in the media (*Figure 2E,F*). The concentration of 100 µM was chosen for the subsequent reversal experiments of AA effects.

Involvement of the NF-κB pathway in the suppression of ACE

In adipose tissue, free fatty acids have been found to activate the RAS through the NF-κB pathway (35). In light of this, we investigated whether inhibiting the NF-κB pathway could rescue the expression of ACE. Compared to control cells, culture of the adipocytes in the presence of the NF-κB

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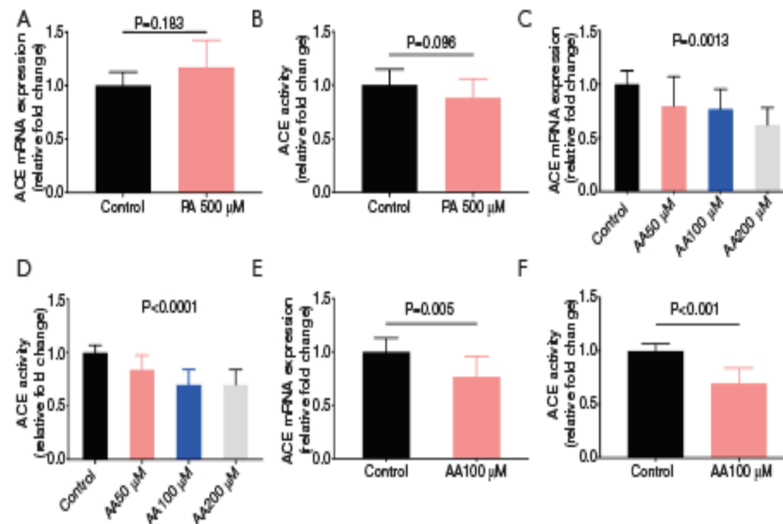

Figure 2 The angiotensin-converting enzyme (ACE) mRNA level in the cell extract and ACE activity in the culture media are measured under the stimuli of palmic acid (PA, 500 μM) or arachidonic acid (AA, 50, 100, 200 μM), respectively. (A) In the presence of 500 μM PA, the mRNA expression of ACE in the fat cell extract is not affected compared with controls (P=0.183). (B) Similar results are obtained for ACE activity in the culture media treated with the same PA concentration (P=0.096). (C) In the presence of AA at different concentrations, the expression of ACE mRNA in the culture media shows a statistical difference (P=0.0013). (D) ACE activity shows a similar response under the same condition (P<0.0001). As the dosage of AA is increased, the ACE expression and activity show trends of decline (n=3). The effect of 100 μM AA on ACE expression and ACE activity are displayed in part (E) and (F), respectively (P=0.005, P<0.001). All values are expressed as mean ± SD of 3 experiments, and each experiment is conducted in triplicate.

pathway inhibitor (BAY117082) increased ACE expression in the presence of AA (*Figure 3A*). In other words, the introduction of the NF-κB inhibitor completely blocked the effects of AA on ACE expression.

20-Hydroxy-5, 8, 11, 14-eicosatetraenoic acid (20-HETE) is a cytochrome P450 (CYP)-derived metabolite of AA which was shown to play a complex role in blood pressure and also blood sugar regulation (36,37). Activation of RAS was reported to be 20-HETE dependent (38,39). The effects from BAY117082 led us to postulate that the response to the NF-κB pathway inhibition might be related to 20-HETE metabolism of AA. We found that the inclusion of 20-HETE inhibitor HET0016 could compromise the effects of AA (*Figure 3B*). Activity of ACE in the media was also elevated compared to that of the AA-treated group (*Figure 3C*). When a mere 10 nM 20-HETE was added in the culture media of adipocytes, ACE activity was suppressed (*Figure 3D*).

Discussion

Dietary fat, in the form of fatty acids, has been found to exert different effects on preadipocyte differentiation and proliferation (40). Excessive saturated fatty acid intake and accumulation have been found to have detrimental effects on metabolism, resulting in insulin resistance, obesity, vascular disease, and hypertension. Obesity is known to activate the RAS pathway (41), and we previously reported that the introduction of a diet high in saturated fat elevates ACE even in normal weight healthy young study participants and leads to increases in blood pressure and blood glucose levels (9,10). The adipose tissue ACE gene expression was increased significantly in response to 6 weeks of a high fat diet, and energy consumption from total fat and saturated fat was 45% and 18%, respectively. We assumed that the high fat diet activated the renin-angiotensin system with increased ACE expression in human adipose tissue. In this study, PA stimulation seemed

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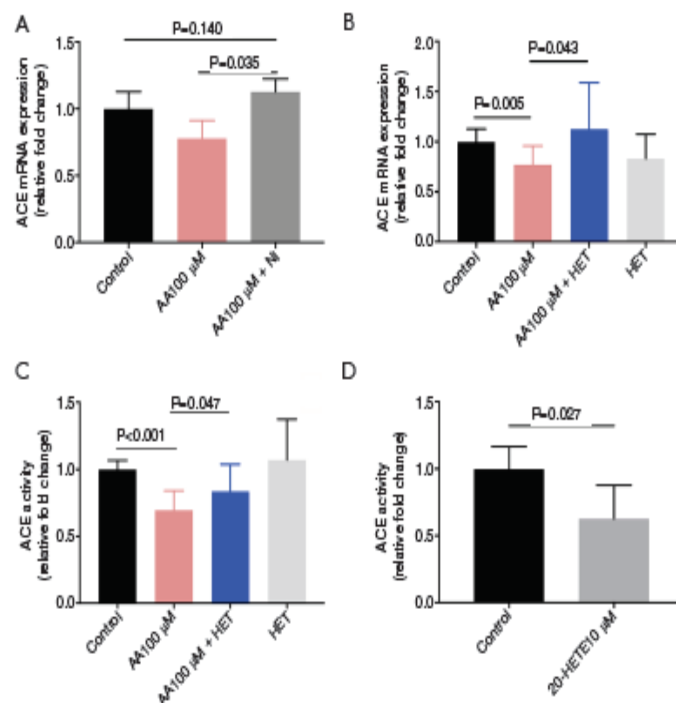

Figure 3 The inhibitory effect of arachidonic acid on ACE production in human subcutaneous adipocytes was reversed by NF-κB inhibitor (NI) and 20-HETE inhibitor HET0016 (HET). (A) The reversal of the inhibition of angiotensin-converting enzyme (ACE) mRNA expression in the presence of 100 μM arachidonic acid (AA100) by the 5 μM NF-κB inhibitor BAY117082 (NI) in cultured adipocytes (n=3). (B) 10 μM 20-HETE inhibitor HET0016 (HET) competes off the inhibition of ACE mRNA expression by 100 μM AA (AA100) (n=3). (C) Reversal of the AA-induced (100 μM, AA100) inhibition of ACE activity by 10 μM HET0016 (HET) (n=3). (D) ACE activity is down-regulated when a mere 10 nM 20-HETE is added in the culture media of adipocytes. All values are expressed as mean ± SD of 3 experiments, and each experiment is conducted in triplicate.

to have no effect on the cultured adipocytes (*Figure 2*). This finding might be ascribed to the fact that saturated fatty acids need to be deposited in adipocytes in an esterified form. The data suggests that the increase in ACE seen in the presence of a high fat diet is not a direct consequence of the interaction of PA with fat cells.

AA is a typical n-6 polyunsaturated fatty acid found in daily food which can be generated by elongation and desaturation from linoleic acid, a predominant n-6 fatty acid in some plant oils, although increased intake may not necessarily result in increased levels of AA in humans (42). It was reported to stimulate preadipocyte differentiation in a cyclooxygenase-dependent manner (43). In this assay, AA was selected as the example of an unsaturated fatty acid to stimulate adipocytes. We found that in the presence of AA, cultured adipocytes exhibited lowered expression of ACE (*Figure 2E*) which, in turn, resulted in the lowered ACE activity in the media and might be considered a protective effect (*Figure 2F*).

Free AA can be metabolized through four enzymatic and one non-enzymatic pathway. The enzymatic pathways include cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP 450). The CYP 450 pathway involves two enzymes: CYP450 epoxygenase, and CYP450 ω-hydroxylase, giving rise to EETs and 20-HETE, respectively (31,44). Other metabolites include prostaglandins (PGs), prostacyclin, thromboxane (Tx), hydroperoxyeicosatetraenoic acid (HPETE), leukotrienes (LTs), lipoxins, hypoxins, and anandamide. The non-enzymatic pathway is important for the production of

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isoprostanes and nitroeicosatetraenoic acid. Most of the AA metabolites are highly bioactive and involved in various crucial vital processes.

20-HETE induces oxidative stress, increases BMI, and is related to metabolic syndrome. In human mesenchymal stem cell-derived adipocytes, the expression levels of major 20-HETE synthases, CYP4F2, decreased during adipocyte differentiation which means lower levels of endogenous 20-HETE exist; however, exogenous administration of 20-HETE (0.1–1 µM) increased adipogenesis in a dose-dependent manner (45). 20-HETE has been proven to be one of the activators of NF-κB (46). In this study, we found that blocking the 20-HETE synthesis using HET0016 reversed the AA-suppressed ACE expression and ACE activity (*Figure 3A,B,C*), and subsequently, we found that adding a mere 10 nM 20-HETE into the culture media down-regulated the ACE activity of unpretreated adipocytes (*Figure 3D*). Thus, we postulate that AA exerts at least part of its ACE-suppressing effect through its metabolite 20-HETE, and inhibiting 20-HETE synthesis can reverse the ACE-suppressing effect. This is not in accordance with a previous report which reported that 20-HETE activates ACE expression through the NF-κB pathway (39). Thus, in this study, a NF-κB inhibitor was also employed to block the activation of this pathway. Clearly, the decreased expression of ACE was also reversed by this perturbation (*Figure 3*).

Notably, our findings have been obtained in cultured human primary adipocytes, therefore differences might be due to tissue and cell type specificities. Human preadipocytes have been shown no differences between DM and non–diabetes mellitus (NDM) patients in accumulating cytoplasmic lipid and upregulating expression of adipogenic genes (47). We can not exclude that the morbid patients undergo epigenetic changes due to metabolic conditions. On the other hand, we suppose that the effects of fatty acids on ACE would be more expressed in adipocytes from obese patients and patients with T2D. Due to the high costs and complexity of this kind of cell culture, ASCs of 3–4 patients are usually used to investigate the pathophysiological mechanisms (48).

Previous reports utilized endothelial cells or adipocytes from other tissues. To our knowledge, this is the first use of adipocytes originating from human subcutaneous stem cells to evaluate the effects of AA relevant to the RAS. In addition, the ASCs were isolated by biopsies from four morbidly obese patients who each had a BMI above 40 kg/m^2, potentially resulting in an abnormal cell state. These ASCs may also be influenced by obesity-related sources in terms of differentiation and physiological function.

Even for adipocytes, the regulation of RAS was reported to be depot specific. For example, according to some reports, angiotensin can only increase fat mass, fat cell sizes, adipose and systemic inflammation in visceral adipose depots but not in subcutaneous depots (6). Thus, the findings described in this study might imply that cell type selection is crucial to data interpretation in the study of RAS functions. However, the current findings may be limited by the cell culture study, and more animal or human studies are needed for confirmation.

The result suggests that the inflammatory NF-κB pathway exerts beneficial effects by lowering ACE expression. Indeed, postprandial inflammation is a common phenomenon and may exert beneficial regulatory effects in adipose tissue as suggested by previous studies (49). Indeed, we observed long-term metabolic improvements upon increasing dietary intake of n-6 fatty acids despite acute increases in adipose inflammatory responses (50). Moreover, elevated levels of AA are not linked to increased risks of cardiovascular disease in large epidemiological meta-analyses (51). Our data, therefore, may help to explain the long-term beneficial effects of increased intake of n-6 fatty acids (52).

The limitations of this study relate to the *in vitro* nature of the investigation which differs from an *in vivo* situation. Moreover, the primary adipocytes were obtained from four morbidly obese individuals and may have undergone epigenetic changes due to the metabolic condition of the patients. Also, larger sample size trails will bring more evidence.

Conclusions

This study primarily demonstrated that subcutaneous adipocytes responded differentially to saturated and unsaturated fatty acids in ACE production. We have provided initial evidence that interfering with adipocyte metabolism might be a potential method to modulate the RAS and its subsequent pathological and physiological events.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-7514

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-7514). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This clinical intervention study was approved by the Ethics Committee of the Charité University Medicine in Berlin (Application no. EA4/006/15) in accordance with the Declaration of Helsinki and registered at www.drks.de (DRKS00009509). Informed consent was acquired from each individual prior to surgery.

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Publication 2

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ORIGINAL RESEARCH

American Heart Association | American Stroke Association.

High-Saturated-Fat Diet Increases Circulating Angiotensin-Converting Enzyme, Which Is Enhanced by the rs4343 Polymorphism Defining Persons at Risk of Nutrient-Dependent Increases of Blood Pressure

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***Background*—**Angiotensin-converting enzyme (ACE) plays a major role in blood pressure regulation and cardiovascular homeostasis. Contrary to the assumption that ACE levels are stable, circulating ACE has been shown to be altered in obesity and weight loss. We sought to examine effects of a high-saturated-fat (HF) diet on ACE within the NUtriGenomic Analysis in Twins (NUGAT) study.

***Methods and Results*—**Forty-six healthy and nonobese twin pairs initially consumed a carbohydrate-rich, low-fat diet over a period of 6 weeks to standardize for nutritional behavior prior to the study, followed by 6 weeks of HF diet under isocaloric conditions. After 6 weeks of HF diet, circulating ACE concentrations increased by 15% (P=1.6×10^{-30}), accompanied by an increased ACE gene expression in adipose tissue (P=3.8×10^{-6}). Stratification by ACE rs4343, a proxy for the ACE insertion/deletion polymorphism (I/D), revealed that homozygous carriers (GG) of the variant had higher baseline ACE concentrations (P=7.5×10^{-8}) and additionally showed a 2-fold increase in ACE concentrations in response to the HF diet as compared to non- or heterozygous carriers (AA/AG, P=2×10^{-6}). GG carriers also responded with higher systolic blood pressure as compared to AA/AG carriers (P=0.008). The strong gene-diet interaction was confirmed in a second independent, cross-sectional cohort, the Metabolic Syndrome Berlin Potsdam (MeSyBePo) study.

***Conclusions*—**The HF-diet-induced increase of ACE serum concentrations reveals ACE to be a potential molecular link between dietary fat intake and hypertension and cardiovascular disease (CVD). The GG genotype of the ACE rs4343 polymorphism represents a robust nutrigenetic marker for an unfavorable response to high-saturated-fat diets.

***Clinical Trial Registration*—**URL: http://www.clinicaltrials.gov. Unique identifier: NCT01631123. **(*J Am Heart Assoc.* 2017;6:e004465. DOI: 10.1161/JAHA.116.004465.)**

Key Words: angiotensin-converting enzyme • blood pressure • diet • gene-diet interaction • nutrigenomics genetics

The zinc metallopeptidase angiotensin-converting enzyme (ACE) plays an important role in blood pressure control and cardiovascular homeostasis as a central regulatory enzyme within the renin-angiotensin system (RAS). ACE catalyzes the generation of the vasoconstrictor angiotensin II from inactive angiotensin I and degrades the vasodilator bradykinin. Its pharmacological inhibition represents a standard of care in the therapy of hypertension and related cardiovascular disease (CVD). Carriers of a frequent insertion/deletion (I/D) polymorphism located in the 16th intron of the ACE gene (*Alu* I/D) are characterized by higher ACE levels and have been shown, albeit inconsistently, to be associated

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with higher blood pressure and increased risk for CVD.[1-3] Despite huge interindividual variability in circulating ACE levels depending on the ACE genotype,[4] circulating levels are reported to be stable within given individuals.[5] However, ACE circulating concentrations or activity levels, which are highly correlated,[6,7] were shown to be increased in obesity[8] and decreased by weight loss.[6,9,10] In addition to its predominant expression in pulmonary endothelium as a primary source of circulating levels, ACE is also expressed in adipose tissue[11,12] with increased expression in response to overfeeding.[13]

We investigated the effects of an isocaloric diet high in total and saturated fat on ACE within the framework of the NUtriGenomic Analysis in Twins (NUGAT) study, which aimed to identify nutrition-responsive genes and biomarkers and their heritability. The cross-sectional Metabolic Syndrome Berlin Potsdam (MeSyBePo) cohort, which includes nutritional assessments, was additionally investigated for reevaluation of gene-diet interactions.

Methods

NUGAT Study

The study protocol was approved by the independent ethics review committee of the Charité-Universitätsmedizin Berlin in accordance with the principles of the Helsinki Declaration of 1975, as revised in 2000. Written informed consent was obtained from all participants prior to the study, which was registered at ClinicalTrials.gov (Unique identifier: NCT01631123).

The study was conducted at the department of Clinical Nutrition at the German Institute of Human Nutrition Potsdam-Rehbrücke. Twin pairs were recruited either from a twin register (HealthTwiSt, Berlin, Germany) or by public advertisements. Exclusion criteria were consumptive diseases, diabetes mellitus, high-grade anemia, renal failure, moderate to severe heart diseases, angina pectoris, or stroke in the last 6 months, food allergy, eating disorders, body weight change ≥3 kg within 3 months prior to the study, pregnancy or breastfeeding, drugs influencing metabolic homeostasis, lipid and liver metabolism, or inflammation (eg, systemic corticosteroids).

Participants were initially screened to determine their eligibility for enrollment in the intervention study. This screening visit comprised physical examination, medical history, anthropometric measurements, and blood analysis. Additionally, a standardized 3-hour, 75-g oral glucose tolerance test (OGTT) was performed. Participants' resting energy expenditure (REE) was measured by indirect calorimetry, and physical activity level (PAL) was assessed by questionnaire to calculate individual daily energy requirements. A total of 46 healthy and nonobese twin pairs, 34 monozygotic and 12 dizygotic, age 18 to 70 years and body mass index (BMI) 18 to 29 kg/m^2 with BMI difference <3 kg/m^2 between twins were included in the study. The CONSORT Flow Diagram is shown in Figure 1. Participants went from a 6-week, carbohydrate-rich, low-fat diet (LF) to standardize for nutritional behavior prior to the study to a 6-week HF diet in a sequential design under isocaloric conditions (Figure 2). Three clinical investigation days (CID) were performed, after 6 weeks of low-fat diet (LF6) and after 1 and 6 weeks of high-saturated-fat diet (HF1 and HF6). At each CID, anthropometric measurements were performed. Three blood pressure readings were taken in a relaxed sitting position with an appropriate size cuff, and the average values were used for analysis. Blood samples were drawn in the fasted state for routine laboratory marker and biomarker analysis in plasma or serum and SNP array-based genotyping. Additionally, a biopsy of the subcutaneous adipose tissue was performed lateral to the umbilicus by needle aspiration for microarray gene expression analysis.

Dietary Intervention

All subjects completed a dietary record for 5 days prior to the study to encompass dietary habits. They commenced with the isocaloric dietary intervention for 6 weeks, receiving a high-carbohydrate, low-fat diet (LF: 55% carbohydrate, 30% fat, 15% protein) in accordance with accepted national dietary guidelines. After the first investigation day (LF6) the diet was changed to a low-carbohydrate, high-saturated-fat diet (HF: 40% carbohydrate, 45% fat, 15% protein) for 6 weeks with emphasis on foods high in saturated fat such as red meat, sausage, bacon, and full-fat dairy products. Participants received a list of 94 food items and individual daily meal plans on how to exchange and combine these foods, and energy intake was adjusted according to body weight if needed. To ensure compliance, participants were given intensive, regular, and detailed dietary guidance by a nutritionist over the entire period of intervention. For 1 week prior to each particular CID, ~70% of the food was provided with detailed daily meal plans to ensure a standardized dietary pattern for all participants. All subjects had to complete 5 dietary records during the 12 weeks of the dietary intervention period.

Blood Parameters

Determination of routine serum parameters (eg, total cholesterol, HDL-cholesterol, triglycerides) was performed using an automated analyzer (ABX Pentra 4000; ABX, Montpellier, France). LDL cholesterol concentrations were calculated using the Friedewald equation. ACE concentrations were measured in the serum of all participants at each CID using a human

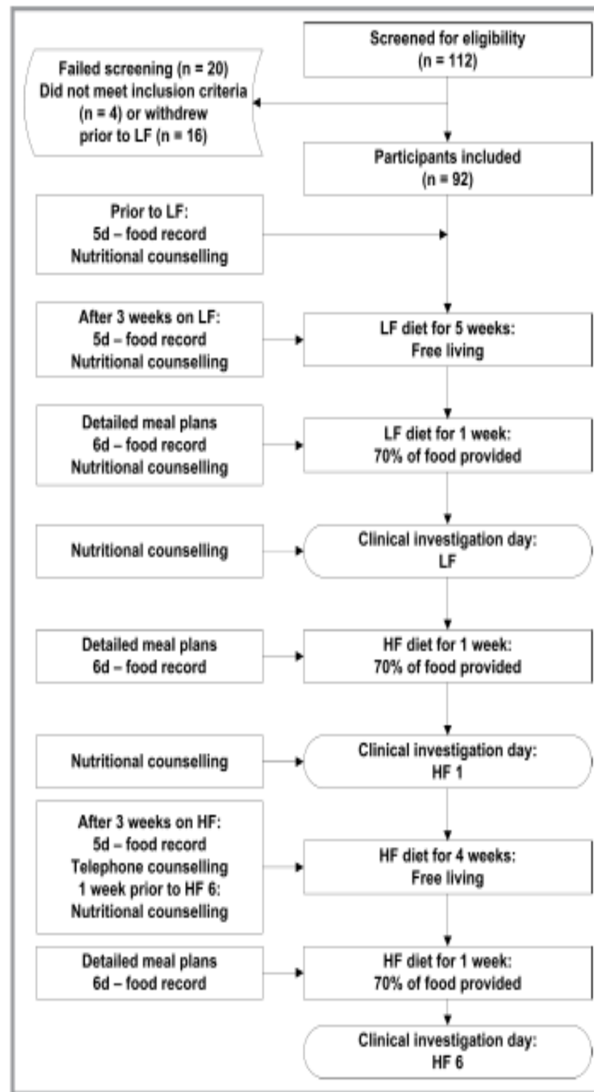

Figure 1. CONSORT flow diagram of the NUGAT study. NUGAT indicates NUtriGenomic Analysis in Twins; HF, high-saturated-fat diet; LF, low-fat diet.

ACE immunoassay ELISA kit (R&D Systems Inc, Minneapolis, MN) with an interassay variance <5%.

Gene Expression Analysis

About 500 mg of subcutaneous adipose tissue were homogenized using a Speed Mill (Analytik Jena, Jena, Germany), and total RNA was extracted by using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quality was assessed by using RNA 6000 Nano-LabChips and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). One microgram of each sample was amplified using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion by Life Technologies, Carlsbad, CA) and hybridized onto Agilent Whole Human Genome 8×60K Gene Expression Microarrays (Agilent Technologies, Santa Clara, CA). Microarray data have been uploaded to NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62199; Accession No: GSE62199). To validate microarray data, quantitative real-time PCR was performed. cDNA was synthesized from 1 μg of RNA of each sample by using the High-Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems by Life Technologies, Carlsbad, CA). Samples were labeled by Power SYBR Green Master Mix and detected

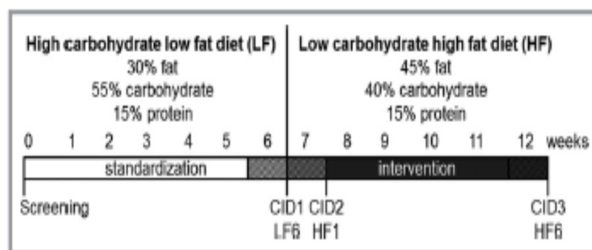

Figure 2. Time line of the NUGAT (NUtriGenomic Analysis in Twins) intervention study. For 1 week prior to each particular investigation day (hatched areas, weeks 6, 7, and 12) most of the food was provided to ensure standardized dietary patterns for all participants. HF1 indicates investigation day after 1 week of the high-saturated-fat diet; HF6, investigation day after 6 weeks of the high-saturated-fat diet; LF6, investigation day after 6 weeks of the low-fat diet.

in triplicates in optical 384-well plates with the ABI ViiA™7 Real-Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA). The samples were normalized to ribosomal protein L32 (RPL32), and the standard curve method was used for evaluation. The primer sequences were ACE forward primer CAAGCACCTGCACAGTCTCAAC, reverse primer TGATCGACGAGGTAGCTGAAGG; RPL32 forward primer CAACGTCAAGGAGCTGGAAGT, reverse primer TTGTGAGCGATCTCGGCAC.

Genotyping

Genomic DNA was isolated from buffy coat using a commercial kit (NucleoSpin, Macherey-Nagel, Düren, Germany) and genotyped on HumanOmniExpressExome BeadChips (Illumina, Inc, San Diego, CA) at the Interdisciplinary Center for Clinical Research (IZKF, Leipzig, Germany).

Genotyping for the Metabolic Syndrome Berlin Potsdam (MeSyBePo) study was performed using a predesigned rs4343 TaqMan SNP Genotyping Assay on 384-well plates using the ABI ViiA™7 Real-Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

Metabolic Syndrome Berlin Potsdam Study

The cross-sectional MeSyBePo study was approved by the ethics commissions of Berlin and Brandenburg, Germany. All individuals gave written informed consent prior to the study. Two thousand three hundred sixty-four white volunteers were randomly recruited from the Berlin and Potsdam areas. Four hundred seventy-nine out of the 2364 participants were excluded from the analyses due to missing data. Dietary data were collected from 671 participants via a 4-day estimated food record that comprised 18 categories with 151 food items. By use of the German Nutrient Database BLS, version 2.3, the mean daily energy and nutrient intakes were calculated. For evaluation of dietary fat intake, data on total fat intake but not on saturated fat were available.

To analyze the influence of a high-fat dietary pattern on ACE rs4343 genotype associations with blood pressure, we excluded misreporting as a main source of error by evaluating under-, normal, and overreporting of energy intake (EI). Therefore, the basal metabolic rate (BMR) was calculated using the Harris-Benedict equation. Based on the ratio of EI to BMR, underreporting, normal, and overreporting of energy intake were defined as <1.35, 1.35 to 2.39 and ≥2.4, respectively.[14]

Statistical Analysis

For estimation of heritability, the "ACE" structural equation model was applied. This covariance analysis relies on comparing the degree of concordance within and between monozygotic versus dizygotic twin pairs and decomposes the proportion of variance into (A) additive genetic influences and (C) common environmental and (E) individual environmental influences. The "ACE" model was calculated using R 2.15.0 plus OpenMX package. Genotype frequencies were analyzed for deviation from the Hardy-Weinberg equilibrium by chi-square test using R 3.1.2 plus Hardy-Weinberg package 1.5.5.

The evaluation of microarray data was performed using Agilent GeneSpring GX Version 11 Software (Agilent Technologies, Waldbronn, Germany). The data set was normalized to LF6, and genes with >1.5-fold changes were analyzed. The statistical significance of expression changes was calculated by one way analysis of variance (ANOVA) and post hoc analysis following a Mann-Whitney U test. *P*-values were adjusted for multiple testing via the Benjamini-Hochberg (FDR) method.

The Kolmogorov-Smirnov test was used to assess variables for normal distribution. Continuous variables with skewed distribution were natural logarithm (ln)-transformed. One-way or repeated-measures ANOVA followed by Bonferroni post hoc test were used to compare mean values for continuous data. To verify significant results for nonnormally distributed

data, the Kruskal-Wallis test was used. Additionally, linear regression analyses were performed with adjustment for main confounders. Correlation analyses were performed using Pearson and Spearman rank correlation coefficients for variables with normal and skewed distributions, respectively. Statistical significance was defined as $P<0.05$. Values are expressed as mean±SD unless otherwise stated. Statistical analyses were carried out using SPSS 20.0 (IBM SPSS, Chicago, IL).

Results

Clinical Characteristics

Table 1 shows anthropometric and clinical characteristics of the 92 healthy and nonobese participants at screening. As calculated from dietary records, energy consumption from total fat/saturated fat was 29%/10% for LF and 45%/18% for HF diet, respectively. Energy consumption from carbohydrates and protein was 55% and 16% for the LF diet and 41% and 15% for the HF diet, respectively. Although the study was performed under isocaloric conditions, the weight of participants increased slightly (0.4±1.0 kg, $P=9\times10^{-6}$). As expected, LDL, HDL, and total cholesterol increased significantly in response to the HF diet ($P_{LDL}=4.7\times10^{-8}$, $P_{HDL}=1.7\times10^{-13}$, $P_{Chol}=8.5\times10^{-11}<0.001$; Table 2), confirming good compliance of the participants with the dietary instructions.[15]

Effect of HF Diet on ACE Serum Concentrations

In response to the HF diet, fasting serum concentrations of ACE increased by 15% (LF6 139±41 ng/mL vs HF6 161±49 ng/mL; repeated-measures ANOVA $P=1.6\times10^{-30}$; Figure 3). Changes in ACE were not influenced by changes in body weight (linear regression, $P=0.114$). At each CID, serum concentrations of ACE were significantly correlated with weight (LF6 $\rho=0.461$, HF1 $\rho=0.474$, HF6 $\rho=0.423$; $P<0.001$), height (LF6 $\rho=0.362$, HF1 $\rho=0.389$, HF6 $\rho=0.354$; $P<0.001$), and thus also with BMI (LF6 $r=0.315$, HF1 $r=0.289$, HF6 $r=0.302$; $P<0.01$), whereas no significant correlations were noted between ACE and systolic or diastolic blood pressure despite a weak correlation between ACE and systolic blood pressure at HF6 ($r=0.234$, $P=0.025$) and between ACE and pulse pressure at HF6 ($\rho=0.228$, $P=0.029$).

Both LDL and total cholesterol were not correlated with ACE concentrations (LF6 $P=0.765$ and $P=0.420$). However, the increase in ACE (ΔACE, HF6-LF6) was modestly but significantly correlated with the increase in LDL cholesterol ($\rho=0.296$, $P=0.004$). Furthermore a modest negative correlation was shown for ACE concentrations and HDL cholesterol (LF6 $\rho=-0.278$, HF1 $\rho=-0.277$, HF6 $\rho=-0.350$; $P<0.01$).

Effect of HF Diet on ACE mRNA Expression in Subcutaneous Adipose Tissue

Adipose tissue ACE gene expression was significantly increased in response to the HF diet (HF6 vs LF6 1.412-fold; ANOVA $P=3.8\times10^{-6}$, post hoc HF6 vs LF6 $P=0.023$, and HF6 vs HF1 $P=0.007$; Figure 4A).

To confirm microarray data, we performed quantitative real-time PCR where ACE mRNA expression was again significantly increased under HF diet conditions (repeated-measures ANOVA, $P=0.005$; Figure 4B).

Table 1. Characteristics of the Participants Overall and Stratified for ACE rs4343 at Baseline in the NUtriGenomic Analysis in Twins Study

	Total	AA Genotype	AG Genotype	GG Genotype	P Value
n	92	31	44	17	0.842
Male/female	34/58	10/21	14/30	10/7	0.120
Age, y	31±14	30±14	31±11	34±20	0.764
BMI, kg/m²	22.8±2.7	22.8±2.2	22.9±2.7	22.9±3.6	0.990
SBP, mm Hg	118±13	116±12	116±14	125±8	0.044
DBP, mm Hg	74±9	73±7	74±9	80±10	0.035
PP, mm Hg	43±9	43±8	42±9	46±10	0.452
Total cholesterol, mmol/L	4.58±0.93	4.55±0.91	4.64±0.93	4.49±1.04	0.822
HDL cholesterol, mmol/L	1.38±0.35	1.38±0.30	1.45±0.39	1.20±0.27	0.043
LDL cholesterol, mmol/L	2.73±0.77	2.73±0.75	2.67±0.76	2.90±0.88	0.600
Triglycerides, mmol/L	0.99±0.44	0.97±0.47	1.01±0.43	0.97±0.39	0.935

Values are shown as mean±SD. BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PP, pulse pressure; SBP, systolic blood pressure.

Table 2. Characteristics of the Participants After the Standardization (LF6) and After 1 and 6 Weeks of High-Saturated-Fat Diet (HF1, HF6)

	LF6	HF1	HF6	P Value
Weight, kg	66.6±11.7	66.5±11.6	67.0±11.8†	<0.001
BMI, kg/m²	22.5±2.7	22.5±2.6	22.6±2.7†	<0.001
SBP, mm Hg	110±12	109±12	110±12	0.681
DBP, mm Hg	70±9	69±9	70±9	0.709
PP, mm Hg	41±9	40±8	40±8	0.899
Total cholesterol, mmol/L	4.29±0.85	4.47±0.87†	4.70±0.91†	<0.001
HDL cholesterol, mmol/L	1.27±0.33	1.32±0.34†	1.41±0.37†	<0.001
LDL cholesterol, mmol/L	2.59±0.71	2.71±0.73*	2.86±0.79†	<0.001
Triglycerides, mmol/L	0.95±0.43	0.89±0.35	0.91±0.37	0.449

Values are shown as mean±SD. BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HF1, investigation day after 1 week of the high-saturated-fat diet; HF6, investigation day after 6 weeks of the high-saturated-fat diet; LDL, low-density lipoprotein; LF6, investigation day after 6 weeks of the low-fat diet; PP, pulse pressure; SBP, systolic blood pressure.
Repeated measures ANOVA with Bonferroni post hoc test: *P<0.05 compared to LF6, †P<0.001 compared to LF6.

Heritability of Serum ACE

Figure 5 shows strong intrapair correlation of circulating ACE concentrations in monozygous twins (LF6 r=0.867, P=3.3×10^{-11}; Figure 5A), whereas intrapair ACE concentrations were not correlated in dizygous twins (LF6 r=0.287, P=0.366; Figure 5B). Based on the "ACE" structural equation model, estimated additive genetic effects contributed to 86%, and shared environmental influences to 14% of the variance of circulating ACE concentrations, respectively. ACE concentrations appeared to be one of the most highly heritable markers in the NUGAT study, with similar heritability estimates seen for bone mineral content (BMC) and height (Figure 5C).

Influence of the ACE rs4343 Polymorphism on ACE Concentrations and Blood Pressure in Response to the HF Diet

Because heritability is linked to genetics, we stratified our analysis by genotypes of the ACE rs4343 variant; due to strong linkage disequilibrium,[16] this serves as a surrogate marker for the ACE I/D polymorphism, where A2350 corresponds to the I-allele and 2350G corresponds to the D-allele.[17,18] Genotype frequencies for ACE rs4343 polymorphism were AA=31, AG=44, and GG=17 and did not deviate

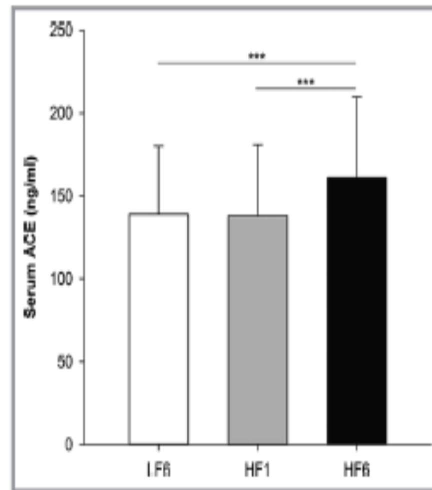

Figure 3. ACE serum concentrations at LF6, HF1, and HF6 (mean±SD; ***P<0.001). ACE indicates angiotensin-converting enzyme; LF6, investigation day after 6 weeks of the low-fat diet; HF1, investigation day after 1 week of the high-saturated-fat diet; HF6, investigation day after 6 weeks of the high-saturated-fat diet.

from values predicted by the Hardy-Weinberg equilibrium (χ^2=0.04, P=0.842). As shown in Figure 6A, ACE serum concentrations significantly differed depending on the genotype, with the lowest concentrations for homozygous noncarriers (AA), intermediate concentrations for heterozygous (AG), and highest concentrations for homozygous carriers of the variant (GG), respectively (ANOVA, LF6 P=4.7×10^{-13}, HF1 P=5.1×10^{-13}, and HF6 P=1.0×10^{-14}). The rs4343 genotype accounted for 47% of the variance in ACE serum concentrations (adjusted R^2=0.466, P=4.2×10^{-14}). After sex (P=4.5×10^{-5}) and BMI (P=0.031) had been included in the model, rs4343 accounted for 60% of the variance (adjusted R^2=0.602, P=3.4×10^{-18}). An increase in ACE serum concentrations in response to the HF diet was confirmed independently of genotypes (repeated-measures ANOVA; AA P=1.6×10^{-11}, AG P=2.6×10^{-18}, and GG P=3.1×10^{-9}), although the increase was doubled for GG carriers compared to AA or AG carriers (Figure 6B; P=2×10^{-6}, P-value adjusted for sex, age, and BMI P_{adj}=1×10^{-5}).

We further performed a bootstrap analysis to exclude bias due to relatedness of twins, randomly assigning both subjects of a twin pair to 1 of 2 data records, and both records were analyzed separately. Random assignment of the twins was repeated 10 000 times to prevent a bias, and group means, standard deviations, and mean P-values were calculated. The increase in circulating ACE levels differed significantly between rs4343 genotypes in both separately analyzed data records (recessive model AA/AG vs GG: 18±1 ng/mL vs 38±4 ng/mL, P_1=0.038 and P_2=0.039).

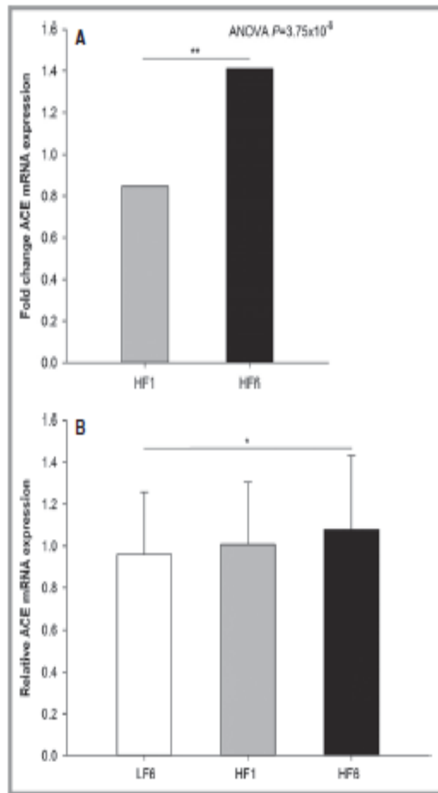

Figure 4. Results of ACE gene expression in subcutaneous adipose tissue by (A) microarray analysis and (B) quantitative real-time PCR. A, Values are presented as fold changes (HF1 vs LF6 and HF6 vs LF6). *P*-values are shown after Benjamini-Hochberg correction (*P<0.05, **P<0.01). B, Values are shown as mean±SD. Bonferroni post hoc test was used to compare main effects (HF6 vs LF6, P=0.010) of repeated-measures ANOVA (P=0.005). ACE indicates angiotensin-converting enzyme; LF6, investigation day after 6 weeks of the low-fat diet; HF1, investigation day after 1 week of the high-saturated-fat diet; HF6, investigation day after 6 weeks of the high-saturated-fat diet.

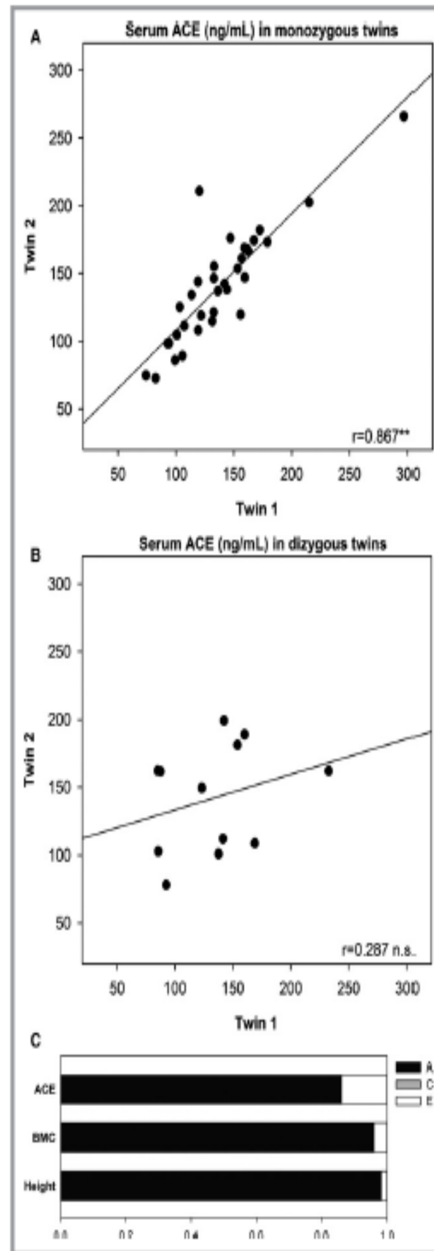

Figure 5. Intrapair correlation of ACE serum concentrations in monozygotic (A) and dizygotic (B) twins (**P<0.01). Estimated heritability (C) for ACE serum concentrations in comparison with estimates for bone mineral content (BMC) and height: "A" additive genetic effects, "C" common environmental influences, and "E" unique environmental influences. ACE indicates angiotensin-converting enzyme.

In Table 1, clinical characteristics at screening are summarized, stratified by genotype. Compared to AA/AG carriers, homozygous carriers of the polymorphism had lower HDL cholesterol (recessive model, P=0.019, P_{adj}=0.028) and higher systolic and diastolic blood pressure (recessive model, P=0.012 and P=0.011, P_{adj}=0.087 and P_{adj}=0.033, respectively). This difference in systolic and diastolic blood pressure did not persist at LF6 (AA/AG vs GG, 109±13 mm Hg vs 115±9 mm Hg, P=0.077, P_{adj}=0.296; 69±9 mm Hg vs 72±7 mm Hg, P=0.154, P_{adj}=0.346) and HF1 (AA/AG vs GG, 109±12 mm Hg vs 113±11 mm Hg, P=0.120, P_{adj}=0.434; 69±9 mm Hg vs 71±8 mm Hg, P=0.304, P_{adj}=0.562). However, after the 6-week HF diet, systolic blood pressure differed significantly between genotypes (AA/AG vs GG, 108±12 mm Hg vs 117±9 mm Hg, P=0.008, P_{adj}=0.033), whereas no differences in diastolic blood pressure were detected (AA/AG vs GG, 69±9 mm Hg vs 73±7 mm Hg, P=0.105, P_{adj}=0.158). Genotype-dependent

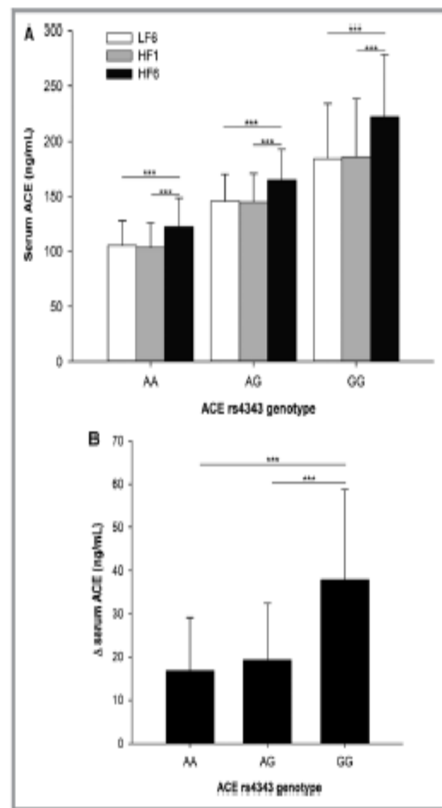

Figure 6. A, ACE serum concentrations at LF6, HF1, and HF6 stratified for ACE rs4343 genotype and (B) ΔACE (HF6–LF6) stratified for ACE rs4343 genotype (mean±SD; ***P<0.001). ACE indicates angiotensin-converting enzyme; LF6, investigation day after 6 weeks of the low-fat diet; HF1, investigation day after 1 week of the high-saturated-fat diet; HF6, investigation day after 6 weeks of the high-saturated-fat diet.

differences in pulse pressure (PP) values failed to reach statistical significance after adjustment for sex, age, and BMI (AA/AG vs GG, 39±8 mm Hg vs 44±7 mm Hg, P=0.026, P_{adj}=0.081).

By performing a bootstrap analysis to exclude bias due to relatedness of twins, we could prove that systolic blood pressure differed significantly between genotypes in both data sets (recessive model: 108±1 mm Hg vs 117±2 mm Hg, P_1=0.039 and P_2=0.038). However, pulse pressure did not differ significantly between genotypes (recessive model: 39±1 mm Hg vs 44±2 mm Hg, P_1=0.179 and P_2=0.176).

Validation of ACE rs4343 Genotype Effects on Blood Pressure in the MeSyBePo Study

A group of 1885 participants (1256 female and 629 male, age 52±14, BMI 29.4±6.3 kg/m^2) of the cross-sectional MeSyBePo cohort were included in the analysis to further validate the interaction of dietary fat intake and ACE rs4343 genotype. ACE rs4343 genotype frequencies were AA=346, AG=1023, and GG=516, which differed significantly from values predicted by the Hardy-Weinberg equilibrium (χ^2=16.77, P=4.2×10^{-5}). Neither systolic nor diastolic blood pressure (SBP, DBP) nor pulse pressure (PP) was significantly associated with ACE genotype (additive model: P_{SBP}=0.524, P_{DBP}=0.391, and P_{PP}=0.309; recessive model P_{SBP}=0.300, P_{DBP}=0.973, and P_{PP}=0.144).

Possible misreporting of dietary intake was evaluated for a total of 671 participants among whom 136 participants (20.3%) underreported, 365 (54.4%) reported normally, and 170 (25.3%) overreported EI.

ACE rs4343 genotype frequencies for participants with plausible reported energy intakes (n=365) were AA=66, AG=197, and GG=102, which did not differ from values predicted by the Hardy-Weinberg equilibrium (χ^2=2.96, P=0.09). ACE rs4343 genotype was again not associated with differences in SBP, DBP, or PP.

To examine the effects of dietary fat intake, we stratified subjects into HF diet consumers with total energy from fat greater than or equal to 37% (representing the average fat intake in Western countries[19]) and normal or LF diet consumers (<37% energy from fat). Mean total fat intake accounted for 41% in the HF group and 31% in the normal/LF group, respectively. Also, in this case, genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (Table 3). For a reported dietary fat intake of <37%, no genotype-specific differences were found, whereas for subjects with dietary fat intake ≥37%, increased systolic blood pressure and pulse pressure values were seen for GG carriers compared to AA/AG carriers (additive model: P_{SBP}=0.011 and P_{PP}=0.023, respectively, Table 3; recessive model: P_{SBP}=0.008, AA/AG vs GG 122±16 mm Hg vs 131±21 mm Hg, respectively; P_{PP}=0.017 AA/AG vs GG 46±12 mm Hg vs 52±14 mm Hg, respectively). In a linear regression model adjusted for sex, age, and BMI, the association of the ACE rs4343 polymorphism with systolic blood pressure and pulse pressure was highly significant (recessive model: β_{SBP}=0.25 and P_{SBP}=0.002; β_{PP}=0.24 and P_{PP}=0.002). This model explained 27% of the variation in systolic blood pressure (r^2_{SBP}=0.270) and 29% of the variation in pulse pressure (r^2_{PP}=0.291). As expected, age (β_{SBP}=0.418 and P_{SBP}=3.45×10^{-7}; β_{PP}=0.469 and P_{PP}=1.09×10^{-8}) and BMI (β_{SBP}=0.208 and P_{SBP}=0.010; β_{PP}=0.179 and P_{PP}=0.025) had a significant effect on systolic and pulse pressure, whereas sex had no effect. The association of the ACE rs4343 polymorphism with systolic blood pressure and pulse pressure remained significant after further adjustment for total energy intake, alcohol intake, smoking, and activity energy expenditure (recessive model: β_{SBP}=0.26 and P_{SBP}=0.002; β_{PP}=0.26 and P_{PP}=0.003).

Table 3. Dietary Fat Modified ACE rs4343 Genetic Effects on Blood Pressure in the Metabolic Syndrome Berlin-Potsdam Study

	Fat Intake <37%				Fat Intake ≥37%			
	AA	AG	GG	*P* Value	AA	AG	GG	*P* Value
n	39	131	68	0.070	27	66	34	0.632
SBP, mm Hg	127±17	123±17	124±19	0.385	126±17	120±15	131±21	0.011
DBP, mm Hg	77±11	78±10	78±10	0.885	77±10	75±8	79±10	0.132
PP, mm Hg	50±11	45±11	46±13	0.061	49±13	45±11	52±14	0.023

Values are shown as mean±SD. ACE indicates angiotensin-converting enzyme; DBP, diastolic blood pressure; PP, pulse pressure; SBP, systolic blood pressure.

Discussion

The NUGAT study demonstrated that circulating ACE concentrations increase in response to a 6-week, high-saturated-fat diet in healthy, nonobese subjects with a parallel increase in adipose tissue ACE mRNA expression. To our knowledge, this is the first study reporting increased circulating ACE concentrations independent of body weight gain or obesity. Thus, next to the well-known HF diet-induced increases in LDL-cholesterol as an established CVD risk factor, we identified ACE as a second parameter that is closely linked with cardiovascular risk and that increases in response to HF diets.

Perhaps even more importantly, we identified the ACE rs4343 polymorphism as a strong nutrigenetic marker that powerfully modulates the extent of HF diet-induced increases in ACE with parallel increases in blood pressure even in healthy, nonhypertensive subjects. The frequent ACE rs4343 variant might therefore be linked to an increased risk of cardiovascular disease, whereas ACE itself might constitute a molecular link between total and saturated fat intake and cardiovascular disease. Thus, our study shows that a nutrigenetic approach offers real potential for providing personalized nutritional advice for disease prevention.

The strong gene-diet interaction we have identified may help to explain inconsistent results in the relationship between ACE levels or ACE genotype associations and blood pressure and CVD.[1-3,20-24] The assumption that ACE levels are associated with blood pressure is due to the facts that ACE catalyzes the production of angiotensin II and ACE inhibitors effectively reduce blood pressure; however, this is not consistently supported by all studies.[7,25] A stratification of total and saturated fat intake by ACE genotype would be expected to improve the risk estimates obtained in these studies. Remarkably, a recent epidemiological meta-analysis did not observe an association between saturated/high fat intake and risk of cardiovascular disease[26] and therefore requested a change in the dietary recommendations of the American Heart Association. This data set should be particularly suitable for assessing the interaction of ACE rs4343, diet, and cardiovascular risk.

With an estimated heritability of 86%, ACE appears to be 1 of the most heritable markers in our study, proving a high genetic component in ACE concentrations. This heritability estimate is considerably higher than the moderate values being reported by both a family-based and twin-based study[27,28] but is most likely explained by the controlled diet conditions underlying our study and might, therefore, be more accurate. Moreover, our study possibly increased the concordance among the monozygous twin pairs by excluding twins with significant differences in body weight.

A large proportion of the heritable variation of ACE concentrations is strongly linked to the ACE gene, which contains a series of frequent polymorphisms in strong linkage disequilibrium with each other.[17,29] The best known is the ACE I/D polymorphism, which accounts for almost 50% of the interindividual variance in circulating ACE levels and represents a quantitative trait locus (QTL).[1,4,30,31] Numerous studies have explored associations of the ACE I/D polymorphism, albeit inconsistently, with increased blood pressure and increased risk for CVD.[1-3,20-24] The rs4343 SNP that was analyzed in our study is a silent coding SNP expressed at the mRNA level and, due to complete linkage disequilibrium, serves as a surrogate marker for the I/D polymorphism.[16-18] At baseline, homozygous carriers of rs4343 (GG) had higher diastolic and by trend higher systolic blood pressure compared to heterozygous carriers and noncarriers (AA/AG). This difference did not persist after 6 weeks of LF diet. This is most likely due to the fact that blood pressure values improved in response to the LF diet, which was in accordance to general national dietary guidelines. After a challenge with the HF diet, genotype-dependent differences in systolic blood reappeared again at HF6. These results were indicative of an underlying gene-diet interaction. To validate these data, we analyzed associations between rs4343 genotype and blood pressure in a subcohort of the cross-sectional MeSyBePo study for whom reliable nutritional data were available. Indeed, whereas no associations between genotype and blood pressure were detected for the whole cohort or for subjects with normal fat intake, significant genotype differences were found in subjects with high dietary fat intake, with the GG carriers having significantly higher

systolic blood pressure and pulse pressure in comparison to AA/AG carriers. Thus, we provided an independent confirmation of the gene-diet interaction.

The mechanism by which diets high in total and saturated fat signal increases in ACE levels is unclear, but it was shown that a diet with high lipid content activates the renin-angiotensin system with increased ACE expression in adipose tissue in mice.[32] The high-lipid diet used was based on soy bean oil, which is rich in unsaturated fatty acids, and therefore stands in contrast to high-fat diets with characteristic high content of saturated fat. However, palmitic acid, the major saturated fatty acid, was shown to induce activation of the renin-angiotensin system in 3T3-L1 adipocytes through toll-like receptor 4 and NF-κB signaling.[33]

A limitation of our NUGAT intervention study is the moderate number of participants with respect to genotype-related data analysis. Nevertheless, we reduced confounding factors by including only metabolically healthy, nonobese, and rather young participants in the study, and finally, we demonstrated that a healthy cohort might be favorable to study gene-diet interactions that affect metabolic and/or cardiovascular risks. Nevertheless, the results might be different in obese subjects, and the results may not be applicable in other ethnicities because ACE genotypes are more variable in nonwhites.[34]

Furthermore, nutritional intake information of our MeSyBePo study cohort is limited in that only data on total fat intake but not on saturated fat were available. Nevertheless, consuming high-fat diets typically stands for increased saturated fat intake.

Our data suggest that a high total and saturated fat intake alters concentrations of ACE in a nutrigenetic manner and provides a potential pathway through which high intake of total and saturated fats contributes to the pathogenesis of cardiovascular diseases. Presumably, dietary strategies to lower LDL cholesterol, which are reducing dietary total and saturated fat (DASH diet, Dietary Approaches to Stop Hypertension[19]), are equally efficient in reducing ACE concentrations. Next to lowered LDL cholesterol concentrations, reduced ACE concentrations might contribute concomitantly to beneficial effects.

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Disclosures

None.

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Publication 3

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Dietary Fat Intake Modulates Effects of a Frequent *ACE* Gene Variant on Glucose Tolerance with association to Type 2 Diabetes

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The frequent *ACE* insertion/deletion polymorphism (I/D) is, albeit inconsistently, associated with impaired glucose tolerance and insulin resistance. We recently observed an enhanced upregulation of ACE by elevated fat intake in *GG*-carriers of the I/D-surrogate rs4343 variant and therefore investigated its potential nutrigenetic role in glucose metabolism. In this nutritional intervention study 46 healthy and non-obese twin pairs consumed recommended low fat diets for 6 weeks before they received a 6-week high fat (HF) diet under isocaloric conditions. Intravenous glucose tolerance tests were performed before and after 1 and 6 weeks of HF diet. While glucose tolerance did not differ between genotypes at baseline it significantly declined in *GG*-carriers after 6 weeks HF diet ($p = 0.001$) with higher 2 h glucose and insulin concentrations compared to *AA/AG*-carriers ($p = 0.003$ and $p = 0.042$). Furthermore, the gene-diet interaction was confirmed in the cross-sectional Metabolic Syndrome Berlin Potsdam study ($p = 0.012$), with the GG-genotypes being significantly associated with prevalent type 2 diabetes for participants with high dietary fat intake $\geq$37% (*GG* vs. *AA/AG*, OR 2.36 [1.02–5.49], $p = 0.045$). In conclusion, the association between the rs4343 variant and glucose tolerance is modulated by dietary fat intake. The *ACE* rs4343 variant is a novel nutrient-sensitive type 2 diabetes risk marker potentially applicable for nutrigenetic dietary counseling.

A large body of evidence supports the role of the renin-angiotensin system (RAS) in modulation of glucose metabolism and its involvement in insulin resistance[1]. Inhibition of angiotensin-converting enzyme (ACE), and thereby antagonizing the RAS, has been shown to improve glucose homeostasis and, albeit inconsistently, to reduce incidence of type 2 diabetes mellitus in large-scale clinical trials[1–7].

Furthermore, the D allele of the frequent *ACE* insertion/deletion (I/D) polymorphism, which is characterized by the presence (I) or absence (D) of a 287-bp *Alu* repeat sequence in the 16th intron of the *ACE* gene, was associated with decreased insulin sensitivity and impaired glucose tolerance in a healthy cohort[8].

Circulating ACE levels were shown to increase in obesity and to decrease during weight loss[9–12]. Recently, we identified *ACE* as nutrition-responsive gene in the NUtriGenomic Analysis in Twins (NUGAT) study, with increased ACE concentrations in response to an isocaloric high fat diet in healthy and non-obese subjects independent of weight gain[13]. Therefore, we intended to investigate potential effects of a high fat diet on glucose tolerance as well as insulin sensitivity in our NUGAT study dependent on the frequent *ACE* rs4343 variant, a surrogate

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	Total	*AA* Genotype	*AG* Genotype	*GG* Genotype	*p*-Value
n	92	31	44	17	0.842
Male/Female	34/58	10/21	14/30	10/7	0.120
Age (years)	31 ± 14	30 ± 14	31 ± 11	34 ± 20	0.764
BMI (kg/m^2)	22.8 ± 2.7	22.8 ± 2.2	22.9 ± 2.7	22.9 ± 3.6	0.990
Fasting insulin (mU/l)	5.21 ± 3.68	4.93 ± 3.04	4.91 ± 3.36	6.51 ± 5.23	0.275
Fasting glucose (mmol/l)	4.31 ± 0.43	4.32 ± 0.43	4.26 ± 0.41	4.37 ± 0.51	0.659
HOMA-IR	1.01 ± 0.76	0.95 ± 0.59	0.94 ± 0.65	1.31 ± 1.16	0.204
HbA_{1c} (%)	5.0 ± 0.4	5.1 ± 0.5	4.9 ± 0.3	5.0 ± 0.2	0.128

Table 1. Characteristics of the participants overall and stratified for *ACE* rs4343 at baseline in the NUtriGenomic Analysis in Twins study. Values are shown as mean ± SD.

	LF6	HF1	HF6	*p*-Value
Weight (kg)	66.6 ± 11.7	66.5 ± 11.6	67.0 ± 11.8*	9×10^{-6}
BMI (kg/m^2)	22.5 ± 2.7	22.5 ± 2.6	22.6 ± 2.7*	1.1×10^{-3}
Fasting glucose (mmol/l)	5.22 ± 0.81	5.15 ± 0.62	5.22 ± 0.61	0.550
Fasting insulin (mU/l)	4.71 ± 3.19	5.55 ± 3.66*	5.11 ± 3.52	0.006
HOMA-IR	1.09 ± 0.76	1.29 ± 0.90#	1.21 ± 0.91	0.012
2-h glucose (mmol/l)	4.46 ± 0.69	4.38 ± 0.64	4.43 ± 0.64	0.707
$iAUC_{glucose}$ (mmol l^{-1} min^{-1})	213 ± 71	215 ± 51	219 ± 72	0.548
$iAUC_{insulin}$ (mU l^{-1} min^{-1})	1748 ± 970	1890 ± 1346	1780 ± 1029	0.235

Table 2. Characteristics of the participants (n = 92) after the standardization (LF6) and after 1 and 6 weeks of high-fat diet (HF1, HF6). Values are shown as mean ± SD. Repeated measures ANOVA with Bonferroni *posthoc* test: *$p < 0.001$ and #$p < 0.01$ compared to LF6. iAUC, incremental area under the curve.

marker for the I/D polymorphism, where the A allele corresponds to the I allele and the G allele corresponds to the D allele[14]. Additionally, the cross-sectional Metabolic Syndrome Berlin Potsdam (MeSyBePo) study, which includes nutritional assessments, was analyzed to evaluate the influence of dietary fat intake on the association between *ACE* genotype and type 2 diabetes prevalence in order to complement and validate the analysis in the NUGAT study.

Results

NUGAT study. The main clinical characteristics at screening of the 92 subjects studied are presented in Table 1. Rs4343 genotype frequencies were distributed according to Hardy-Weinberg equilibrium (Table 1). At screening, no significant differences in markers of glucose metabolism and insulin sensitivity were observed between rs4343 genotypes ($p_{fasting\ insulin} = 0.275$, $p_{fasting\ glucose} = 0.659$, $p_{HOMA\text{-}IR} = 0.204$, $p_{HbA1c} = 0.128$, Table 1).

During HF diet intervention (Table 2), fasting glucose values and glucose tolerance as assessed by ivGTT did not change, whereas fasting insulin values (repeated measures ANOVA; $p = 0.006$) and HOMA-IR increased (repeated measures ANOVA; $p = 0.012$) with significant differences in response to 1 week of HF diet (Bonferroni *posthoc* analysis; LF6 (clinical investigation day (CID) after 6 weeks of carbohydrate-rich low-fat diet) vs. HF1 (CID after 1 week of high fat diet): $p_{fasting\ insulin} = 2.4 \times 10^{-4}$, $p_{HOMA\text{-}IR} = 0.002$). However, we observed a significant rs4343 genotype × HF intervention interaction on fasting glucose levels (repeated measures ANOVA, interaction term rs4343 (recessive model) × HF intervention: $p = 0.001$, $p = 0.004$ adjusted for sex, age and BMI). Stratified by rs4343 genotype (recessive model), we observed a significant increase in fasting blood glucose concentrations in *GG*-carriers by 0.5 ± 0.1 mmol/l (mean ± SEM), whereas no change was observed for *AA/AG*-carriers (repeated measures ANOVA; $p_{AA/AG} = 0.191$ *vs.* $p_{GG} = 0.009$; Fig. 1a). Changes in fasting blood glucose in response to the HF diet also significantly differed between genotypes (Fig. 1b, $p = 4.5 \times 10^{-4}$). As shown in Fig. 1c, fasting insulin concentrations significantly increased irrespective of genotypes (repeated measures ANOVA, $p_{AA/AG} = 0.038$ and $p_{GG} = 0.039$; interaction term rs4343 (recessive model × HF intervention: $p = 0.175$), with significant differences in response to 1 week of HF diet (Bonferroni *posthoc* analysis $p_{AA/AG} = 0.008$ and $p_{GG} = 0.005$). However, after 6 weeks of HF diet fasting insulin concentrations were significantly higher in *GG*-carriers compared to *AA/AG*-carriers ($p = 0.042$). A significant interaction between rs4343 genotype and the HF intervention was also observed on HOMA-IR (repeated measures ANOVA, interaction term rs4343 (recessive model) × HF intervention: $p = 0.008$, $p = 0.028$ adjusted for sex, age and BMI). As shown in Fig. 1d, HOMA-IR values increased in *GG*-carriers (repeated measures ANOVA $p_{GG} = 0.002$) with significantly higher concentrations at HF6 (CID after 6 weeks of high fat diet) compared to *AA/AG*-carriers (*AA/AG vs. GG* $p = 0.022$), whereas HOMA-IR measures did not change for *AA/AG* genotypes during HF diet (repeated measures ANOVA $p_{AA/AG} = 0.075$).

A significant genotype × intervention interaction was also revealed for the incremental area under the curve (iAUC) for glucose during ivGTT (repeated measures ANOVA, interaction term rs4343 (recessive model) × HF intervention: $p = 0.014$, $p = 0.025$ adjusted for sex, age and BMI). In response to a 6-week isocaloric high fat diet, incremental area under the curve (iAUC) for glucose during ivGTT significantly increased in *GG*-carriers

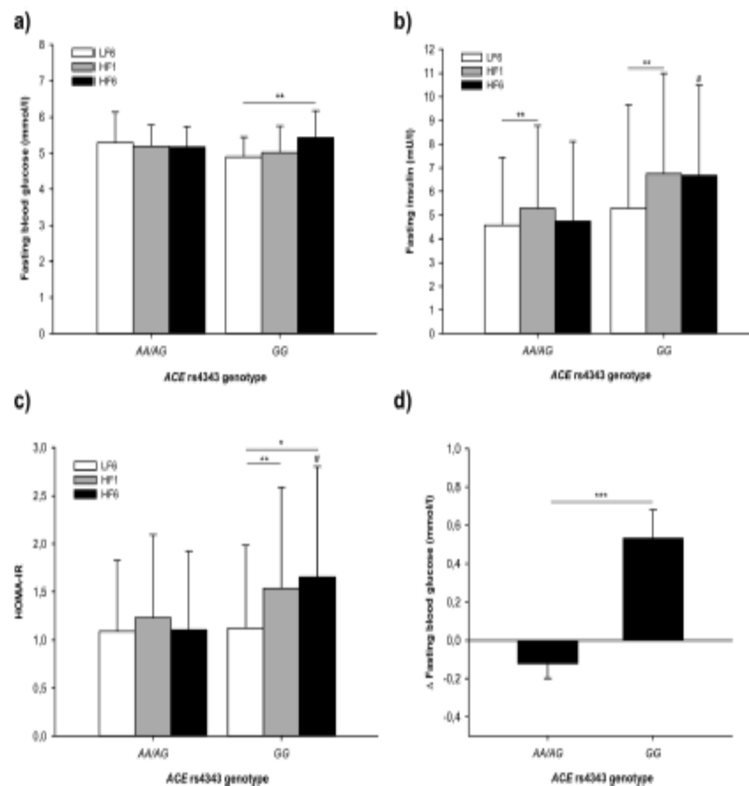

Figure 1. Measured and calculated parameters of glucose metabolism at LF6, HF1 and HF6 stratified by *ACE* rs4343 genotype (recessive model): (**a**) Fasting blood glucose, (**b**) Δ Fasting blood glucose (HF6-LF6; mean ± SEM), (**c**) Fasting insulin and (**d**) HOMA-IR. Data are shown as mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$ vs. *AA/AG* at HF6. LF6: CID after 6 weeks of LF diet; HF1/HF6: CID after 1/6 weeks of HF diet.

(Fig. 2b and c; repeated measures ANOVA, $p_{iAUCglucose} = 0.001$), whereas no change was observed for *AA/AG*-carriers (repeated measures ANOVA, $p_{iAUCglucose} = 0.941$; $\Delta iAUC_{glucose}$ LF6-HF6, *AA/AG* vs. *GG*: $p = 0.009$; *AA/AG* vs. *GG* $iAUC_{glucose}$: $p_{LF6} = 0.330$, $p_{HF1} = 0.999$, $p_{HF6} = 0.028$). iAUC for insulin was also significantly higher in *GG*-carriers after 6 weeks of HF diet compared to *AA/AG*-carriers (Fig. 2d, $p_{iAUCinsulin} = 0.027$). Furthermore, *GG*-carriers responded with higher 2 h glucose levels during ivGTT compared to AA/AG-carriers (*GG*: 4.8 ± 0.7 mmol/l; *AA/AG*: 4.3 ± 0.6 mmol/l; $p = 0.003$).

Indices for insulin sensitivity (Si), glucose effectiveness (Sg) and diposition index (DI) did not differ significantly between the genotypes (*AA/AG* vs. *GG*; Si_{HF6} 11.6 ± 7.7 vs. 8.8 ± 5.3 $(mU/l)^{-1} \times min^{-1}$, $p_{HF6} = 0.204$; Sg_{HF6} 2.09 ± 1.03 vs. 1.97 ± 0.95 × 100 min^{-1}, $p_{HF6} = 0.652$; DI_{HF6} 2759 ± 1708 vs. 3311 ± 2377, $p_{HF6} = 0.317$) and did not change significantly in response to HF diet. The acute insulin response to glucose (AIRg) significantly differed between genotypes at HF1 and HF6 (*AA/AG* vs. *GG*; $AIRg_{LF6}$ 236 ± 118 vs. 298 ± 159 $mU \times l^{-1} \times min$, $p_{LF6} = 0.081$; $AIRg_{HF1}$ 244 ± 111 vs. 340 ± 121 $mU \times l^{-1} \times min$, $p_{HF1} = 0.003$; $AIRg_{HF6}$ 257 ± 140 vs. 363 ± 155 $mU \times l^{-1} \times min$, $p_{HF6} = 0.010$).

Metabolic Syndrome Berlin Potsdam (MeSyBePo) study

To examine dietary fat intake dependent effects, we stratified subjects into high fat diet consumers with energy from total fat ≥37% (cutoff value represents the average fat intake in western countries)[15] and normal/low fat diet consumers (<37% energy from fat). Genotype frequencies did not deviate from Hardy-Weinberg equilibrium (irrespective of fat intake $p = 0.085$, <37% $p = 0.074$, ≥37% $p = 0.584$).

Irrespective of dietary fat intake no association between the rs4343 variant and type 2 diabetes was found (recessive model: OR 1.11 [0.61–2.02], $p = 0.736$ adjusted for age, sex and BMI). After stratification by fat intake, a significant association of rs4343 polymorphism with type 2 diabetes was observed in subjects with a reported dietary fat intake ≥37% with a 2.7-fold higher risk for type 2 diabetes for *GG*-carriers as compared to *AA/AG*-carriers ($p = 0.035$ after adjustment for age, sex and BMI), whereas for normal fat intake no association was found (Table 3). Furthermore, *GG*-carriers with high dietary fat intake had 4.6-fold increased risk for type 2 diabetes as compared to *GG*-carriers with normal dietary fat intake (recessive model: OR 4.61 [1.58–13.46],

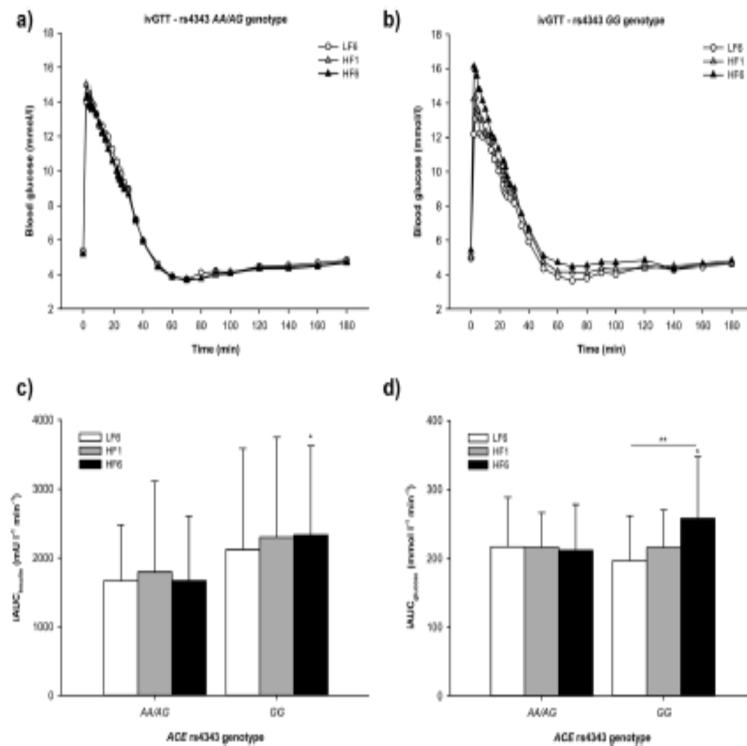

Figure 2. Blood glucose concentrations during ivGTT in (**a**) *AA/AG*-carriers and (**b**) *GG*-carriers. Increments of (**c**) blood glucose and (**d**) insulin at LF6, HF1 and HF6 (mean ± SD; *$p < 0.05$ vs. *AA/AG*-carriers at HF6).

	Fat intake < 37%		Fat intake ≥ 37%	
	AA/AG	*GG*	*AA/AG*	*GG*
$n_{control}/n_{case}$	146/27	64/8	80/20	23/13
OR (95% CI)	1 (ref.)	0.60 (0.25–1.47)	1 (ref.)	2.71 (1.07–6.84)
p value	—	0.267	—	0.035

Table 3. Association between *ACE* rs4343 genotype and type 2 diabetes in the cross-sectional MeSyBePo cohort Binary logistic regression model adjusted for age, sex and BMI. $n_{control}/n_{case}$, participants without/with type 2 diabetes.

$p = 0.005$ adjusted for age, sex and BMI). Also a multiplicative interaction term between genotype and dietary fat intake was significant (recessive model: OR 1.45 [1.09–1.94], $p = 0.012$ adjusted for age, sex and BMI).

Discussion

The results of the NUGAT study showed that dietary fat intake decisively modulated the association of the *ACE* rs4343 variant with impaired glucose metabolism and insulin resistance in healthy and non-obese subjects. Furthermore, we observed in the cross-sectional MeSyBePo cohort that the association between the rs4343 variant and prevalent type 2 diabetes was also influenced by dietary fat intake, with an increased risk for type 2 diabetes in *GG*-carriers of the rs4343 polymorphism provided that dietary fat intake was ≥37%.

The rs4343 variant is in nearly perfect linkage disequilibrium with the *ACE* I/D polymorphism[16], whereby the A-allele corresponds to the insertion (I) variant and the G-allele to the deletion (D) variant, which is characterized by higher circulating enzyme levels[17]. With regard to associations between the *ACE* I/D polymorphism and glucose metabolism, results have been contradictory. While in a study of Bonnet *et al.* healthy subjects homozygous for the D-allele were shown to have decreased insulin sensitivity as measured via clamp and increased 2 h plasma glucose concentrations during OGTT, they and others reported no association between the *ACE* I/D variant and fasting concentrations of insulin and glucose in non-diabetic subjects[8, 18, 19]. Studies investigating the association of the I/D variant with risk for type 2 diabetes have also yielded conflicting results[20–22]. However, two meta-analyses demonstrated an increased type 2 diabetes risk for the D-allele[23, 24]. Also treatment with ACE inhibitors is associated with improvements in glucose metabolism and, albeit inconsistently, reduced incidence of mellitus in large-scale clinical trials[1–7].

With regard to our observations in healthy subjects in the NUGAT study, we found no association per se between glucose tolerance assessed as increments of glucose and insulin during ivGTT as well as fasting glucose and insulin concentrations confirming previous studies with regard to the I/D variant. Nevertheless, after 6 weeks of challenge with a HF diet significantly impaired values for glucose and insulin concentration could be observed, demonstrating that a nutritional challenge influences the effect of the genotype on those parameters. Also in our second, independent MeSyBePo cohort, type 2 diabetes was more prevalent among *GG*-carriers given that dietary fat intake was high. Our results show that consideration of dietary fat intake may be required to elucidate effects of *ACE* rs4343 on glucose metabolism and may explain inconsistent results of previous studies.

The NUGAT study is limited by the relatively small number of participants with respect to genotype-stratified data analysis. Nonetheless, confounding was reduced by including only metabolically healthy, normotensive, non-obese and rather young participants into the study. Moreover, all participants were Caucasians; therefore applicability to other non-Caucasian ethnicities needs to be elucidated. In general, the molecular mechanism by which altered ACE concentrations, *ACE* genotype and ACE inhibitors affect glucose metabolism require clarification, as they are poorly understood. A broadly based understanding will create the basis to elucidate the interplay between ACE and dietary fat and its impact on glucose metabolism. Another aspect which deserves further investigation is the quality of fat. The high fat dietary pattern in the NUGAT study was characterized by the emphasis on saturated fats (18% of 45% energy from total fat) from meat and whole-milk products. Dairy fat intake was inversely associated with markers of glucose metabolism in a Swedish study[25]. Therefore, it would be important to evaluate the effects of fat quality on the gene-diet interaction.

Our data showed that markers of glucose metabolism and type 2 diabetes risk were significantly influenced by interactions between the *ACE* rs4343 variant and dietary fat intake and suggested that homozygous carriers of the G-allele responded unfavorably to high fat diets with increased risk for altered glucose metabolism and type 2 diabetes.

Methods

NUGAT study design. The NUGAT study was approved by the independent ethics review committee of the Charité-Universitätsmedizin Berlin and conducted in accordance with the principles of the Helsinki Declaration of 1975, as revised in 2000. All participants provided written informed consent prior to the study.

Details of recruitment and phenotyping of study participants as well as dietary interventions were published recently[13]. 46 healthy and non-obese twin pairs (34 mono- and 12 dizygotic pairs; 58 female and 34 male subjects) with a mean age of 31 ± 14 years and a mean BMI of 22.8 ± 2.7 kg/m^2 were included in the study. At screening a standardized 3 h, 75 g OGTT (oral glucose tolerance test) with insulin measurements was performed.

The dietary intervention was carried out in a sequential design and under isocaloric conditions. Individual energy requirements were calculated based on participants resting energy expenditure (REE) determined by indirect calorimetry and physical activity level assessed by questionnaire. Participants were standardized for their nutritional behavior prior to the study via a 6-week carbohydrate-rich low-fat diet (LF, 55% carbohydrate, 30% fat, 15% protein) before they switched to a 6-week HF diet (40% carbohydrate, 45% fat, 15% protein) with emphasis on foods high in saturated fat. Participants were given intensive, regular and detailed dietary guidance by a nutritionist over the entire period of intervention to ensure compliance. Furthermore, all participants had to complete 5 dietary records during the 12 weeks of the dietary intervention period. Dietary protocols had been analyzed via Software PRODI 4.5 LE 2001 Expert (Firma Nutri-Science, Hausach, Germany) to quantify energy and macronutrient composition to ensure the adherence to the dietary phases.

Clinical investigation days (CIDs) were performed after 6 weeks of LF diet (LF6) and after 1 and 6 weeks of HF diet (HF1 and HF6). At each CID fasting blood glucose and insulin concentrations were measured and intravenous glucose tolerance tests were performed.

Intravenous glucose tolerance test (ivGTT). After 12 hours of fasting a bolus of glucose (11.4x body surface area/0.5 ml) was infused as a 50% (w/v) solution (Braun, Melsungen, Germany) intravenously within 1 min. Afterwards physiologic saline solution was injected in order to prevent phlebitis. After 20 min, an insulin bolus of 0.03 U per kg body weight was injected (InsumanRapid 40 IU/ml, Sanofi-Aventis, Frankfurt, Germany). Blood for measurement of insulin and glucose concentration was drawn from a peripheral venous access catheter placed into a forearm vein at 0, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160 and 180 min.

We applied the linear trapezoid rule to calculate incremental areas under the curve (iAUC) for plasma glucose and insulin. Indices for insulin sensitivity (Si), glucose effectiveness (Sg), acute insulin response to glucose (AIRg) and disposition index (DI) were calculated using the minimal model (MINMOD Millenium)[26].

Blood Parameters. Blood glucose was measured by the glucose oxidase method (Super GL, Dr. Müller Gerätebau, Freital, Germany). Insulin was determined in serum by ELISA (Mercodia, Uppsala, Sweden). Homeostasis model assessment of insulin resistance (HOMA-IR; [(fasting insulin (mU/l)) × (fasting glucose (mmol/l))/22.5]) was calculated to determine basal/hepatic insulin sensitivity.

Rs4343 polymorphism genotyping. In the NUGAT study, genomic DNA was extracted from buffy coat samples (NucleoSpin, Macherey-Nagel, Düren, Germany) and genotyped using HumanOmniExpressExome BeadChips (Illumina, Inc., San Diego, CA, USA) at the Interdisciplinary Center for Clinical Research (IZKF, Leipzig, Germany). For the Metabolic Syndrome Berlin Potsdam study, genotyping was performed using a predesigned TaqMan SNP Genotyping Assay (ViiA7 System; Applied Biosystems, Foster City, CA).

Metabolic Syndrome Berlin Potsdam Study. The cross-sectional Metabolic Syndrome Berlin Potsdam (MeSyBePo) study was approved by the ethics commissions of Berlin and Brandenburg, Germany. All participants provided written informed consent.

The study included 2364 participants, who were randomly recruited from Berlin, Potsdam and surroundings, Germany. All participants underwent physical examination and fasting blood test, as well as a standardized 3-h 75-g OGTT (unless no evident diagnosis of diabetes was existent) with insulin measurements in nondiabetic subjects. Diabetes was diagnosed considering the ADA criteria from 2002[27]. Dietary information was assessed by a 4-day estimated food record which comprised 18 categories with 151 food items. Means of daily energy and nutrient intakes were calculated on the basis of the German Nutrient Database BLS version 2.3. Misreporting of dietary intake was evaluated as recently published[13] and only subjects who reported normal energy intakes were included in the analyses. A complete set of data including dietary information (given normal dietary reporting) was available from 381 participants (305 females and 76 males, mean age 53.4 years, mean BMI 28.7 kg/m^2).

Statistical Analysis. Variables were assessed for normal distribution using the Kolmogorov-Smirnov test and were natural logarithm (ln)-transformed in case of skewed distribution. To compare mean values for continuous data one-way or repeated measures ANOVA followed by Bonferroni *posthoc* test was used. Analyses were adjusted for potential confounding variables such as sex, age and BMI. Significant results for non-normally distributed data were verified using the Kruskal-Wallis test as non-parametric equivalent of the ANOVA.

Genotype frequencies were analyzed for deviation from Hardy-Weinberg equilibrium by chi-square test using R 3.1.2 plus HardyWeinberg package 1.5.5. Chi-Square test was used to study associations between rs4343 genotype and type 2 diabetes frequencies. To estimate odds ratios in the MeSyBePo cohort unconditional logistic regression analysis was performed, also with adjustment for age, sex and BMI. Statistical significance was designated at $p < 0.05$. Values are expressed as mean ± SD, unless otherwise stated. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

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Author Contributions

R.S. researched data, substantially contributed to analysis and interpretation of the data and drafted the manuscript. M.A.O. and T.F. performed statistical analysis and contributed to the discussion and critically reviewed the manuscript. J.S., M.M., D.S., R.N.B., L.X., A.-C.S. and S.K. contributed to data acquisition and interpretation and critically reviewed the manuscript. S.H. and M.K. contributed to study design, subject recruitment, data collection and reviewed the manuscript critically. A.F.H.P. conceived, designed and supervised the NUGAT study and contributed to data interpretation and critical review of the manuscript. All authors gave their final approval of the version of the manuscript to be published.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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Complete list of Publications

Original papers

1- **Xu L**, Schüler R, Xu C, Seebeck N, Markova M, Murahovschi V, Pfeiffer AFH. Arachidonic acid inhibits the production of angiotensin-converting enzyme in human primary adipocytes via a NF-κB-dependent pathway. Ann Transl Med. 2020 Dec;8(24):1652. doi: 10.21037/atm-20-7514. PMID: 33490164; PMCID: PMC7812212.
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