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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1702017 since 2019-05-15T16:36:52Z

Published version:

DOI:10.14309/ajg.000000000000154

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This is an author version of the contribution published on: Questa è la versione dell'autore dell'opera: The American Journal of Gastroenterology, 114(4), 2019, doi: 10.14309/ajg.0000000000000154 The definitive version is available at: La versione definitiva è disponibile alla URL: <u>https://insights.ovid.com/crossref?an=00000434-900000000-99709</u> http://creativecommons.org/licenses/by-nc-nd/4.0/

Angiotensin II Type 1 Receptor rs5186 gene variant predicts incident NAFLD and associated hypertension.

Role of dietary fat-induced pro-inflammatory cell activation

RUNNING TITLE: AGTR1 and NAFLD

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Word count: text 4545 abstract 248

Tables: 6Figures: 2

Financial support: Grant ex-60%, 2013, Dept of Medical Sciences, University of Turin

Potential competing interests: none

Abstract

Objective. Hypertension has been linked to the presence and severity of nonalcoholic fatty liver disease(NAFLD) through unclear mechanisms. The gain-of-function rs5186 A1166C variant in Angtiotensin Receptor Type 1(AGTR1) gene has been linked to hypertension, cardiovascular disease(CVD) and metabolic syndrome.

We assessed the impact of AGTR1 A1166C variant on NAFLD incidence and severity and on glucose and lipid metabolism and explored underlying mechanisms(s).

Methods. We followed-up 314 healthy nonobese nondiabetic, nonhypertensive, insulin sensitive participants in a population-based study, characterized for AGTR1 rs5186 A1166C variant, adipokine profile, inflammatory and endothelial dysfunction markers.

An independent cohort of 78 biopsy-proven nondiabetic NAFLD patients and controls underwent an OGTT with Minimal Model analysis of glucose homeostasis, and an oral fat tolerance test with measurement of plasma lipoproteins, adipokines, MCP-1, calprotectin, and of Nuclear Factor(NF)-κB activation in circulating mononuclear cells(MNCs).

Results. AGTR1 A1166C polymorphism predicted 9.8-year incident

NAFLD(OR:1.67,95%CI:1.26-2.21), NASH and advanced fibrosis and hypertension (OR:1.49, 95%CI:1.12-2.63). In the cross-sectional cohort, AGTR1 C-allele carriers had higher insulin resistance. Despite comparable fasting lipid profiles, AGTR1 C-allele carriers showed postprandial triglyceride-rich and cholesterol-rich VLDL lipoprotein accumulation, higher resistin, MCP-1 and calprotectin responses and NF-κB activation in MNCs, and a blunted postprandial adiponectin response to fat, which predicted liver histology, hepatocyte apoptosis activation, insulin resistance, and endothelial dysfunction. AGTR1

Conclusion. A1166C variant affects liver disease, insulin resistance and endothelial dysfunction in NAFLD, at least in part by modulating adipokine, chemokine and pro-inflammatory cell activation in response to fat ingestion .

Keywords: AGTR1, NF-KB, postprandial lipemia, lipogenesis, adipokines, glucose homeostasis

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Abbreviations: AI: adaptation Index; AHA: American Heart association; BP: blood pressure; C: cholesterol; CGI: CP-genic Index; CK-18: cytokeratin-18; CRP: C-reactive protein; DI: Disposition Index; Dia: diastolic; HOMA-IR: homeostasis model assessment of insulin resistance; IDF: International Diabetes Federation; ICAM: intercellular adhesion molecule; IGI: Insulinogenic Index; METS: Metabolic equivalent of activity; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; NHLBI: National Heart Lung and Blood Institute; OFTT: oral fat tolerance test; OGTT: oral glucose tolerance test; oxLDL: oxidized Low Density Lipoprotein; PNPLA3: patatin-like phospholipase domain-containing 3; Sys: systolic; T2DM: type 2 diabetes mellitus; Tg: triglycerides;

STUDY HIGHLIGHTS

What is current knowledge

- Hypertension is associated with the presence and severity of NAFLD, but connecting mechanisms remain elusive. The renin-angiotensin system (RAS) has been experimentally linked to both hypertension and liver injury.
- We assessed the impact of the gain-of-function AGTR1 A1166C variant on NAFLD incidence and severity and explored potential mechanisms(s).

What is new here

- AGTR1 A>C variant predicted NAFLD incidence and severity and NAFLD-associated hypertension
- *AGTR1* A>C variant modulated postprandial lipoprotein metabolism, and acute proinflammatory response to fat ingestion, which may mediate the observed effect of this polymorphism on liver disease
- combining antiotensin receptor blockers with drugs targeting postprandial lipemia, even in the absence of fasting hyperlipidemia, may have synergistic benefits in NAFLD carriers of at-risk AGTR1 allele.

Introduction

Nonalcoholic fatty liver disease (NAFLD) ranges from simple steatosis (SS) to steatosis plus necroinflammation (nonalcoholic steatohepatitis, NASH). NASH is projected to be the leading cause of liver transplantation by 2020 and NAFLD predisposes to cardiovascular disease (CVD) through mechanisms poorly understood(1,2).

Epidemiological data suggest a bi-directional association between NAFLD and blood pressure(3): hypertension has been linked to the presence and severity of NAFLD; conversely, NAFLD has been associated with incident hypertension and endothelial dysfunction(4,5).

Genetic and/or environmental mechanisms linking NAFLD to hypertension are poorly understood, but may involve adipose tissue dysfunction, pro-/anti-inflammatory adipokine imbalance and dysregulated lipid metabolism(6).

Among environmental factors, dietary fat excess has been linked to endothelial dysfunction and cardio-metabolic risk in the general population(7,8), while the association with NAFLD remains controversial(9).

Among genetic modifiers, the single nucleotide polymorphism (SNPs) more robustly associated with NAFLD, are rs738409 in *patatin-like phospholipase-3 (PNPLA3)*, which does not affect cardiometabolic risk, rs58542926 in *Transmembrane 6 superfamily member 2* gene (*TM6SF2*) variant, which is associated with lower hepatic and plasma lipids, and rs641738 in *Membrane Bound O-Acyltransferase Domain Containing 7 gene (MBOAT7)*, which affects insulin resistance mainly through modulating hepatic fat content. Hence, further investigation on genetic and environmental factors promoting liver disease progression and cardio-metabolic risk in NAFLD is warranted(1,10,11).

Growing evidence links renin-angiotensin system (RAS), and especially Angiotensin-II Receptor Type 1 (AGTR1) activation, to hypertension and CVD in the general population and to liver injury in experimental NAFLD(12,13), while observational retrospective data suggest RAS blockers may slow liver fibrosis progression in NAFLD patients(14,15).

The common functional *AGTR1 A1166C polymorphism* (*AGTR1 rs5186 A>C*), consisting of an adenine-to-cytosine transversion at position 1166 in the 3'-untranslated region of the *AGTR1* gene, affects AT1R expression and activity, with C-allele being associated with AGTR1 upregulation(16).

AGTR1 A1166C polymorphism predicts development of hypertension(17), CVD(18), and metabolic syndrome(19), and influences response to RAS blockers and to other antihypertensive medications in patients at high CVD risk(20,21).

AGTR1 gene is highly expressed in adipose tissue (x7.4), liver (x7.0), leukocytes (x4.5), and intestine (x4.3)(22). On this basis, we hypothesized *AT1R A1166C* polymorphism may affect the risk of developing NAFLD and NAFLD-associated hypertension and affect liver disease severity, we:

1)longitudinally assessed the impact of *AT1R A1166C* variant on NAFLD incidence and on NAFLDassociated hypertension in initially healthy, insulin sensitive, nonhypertensive, nondiabetic subjects without metabolic syndrome.

2)investigated mechanisms connecting *AT1R A1166C* variant to liver injury, glucose and lipid metabolism in an independent cohort of biopsy-proven NAFLD patients and healthy controls.

Methods

Subjects. Based on available data showing a *AGTR1 A1166C* C allele frequency of 28% and a prevalence of NAFLD of 30%(1,17,18,23), assuming an effect size of \geq 1.8(OR for NAFLD across genotypes) and allowing for a 10% drop-out rate, at least 306 subjects were needed to detect a significant (p<0.05) difference in NAFLD incidence across genotypes with a power of 80%. Among 1658 Caucasians aged 45–64 years participating in a metabolic survey in 2004-2005 (previously described)(24), 335 randomly selected subjects gave informed consent to be included in the study. At baseline, all were in good general health, with normal findings on medical history, physical examination, chemical screening battery and abdomen ultrasound. Subjects with one of the following conditions were excluded from the study: hypertension, diabetes, obesity, dyslipidaemia, metabolic syndrome, insulin resistance, CVD, significant alcohol consumption (>20 g/d in males and >10 g/d in females, assessed by a validated questionnaire), known liver disease, ultrasonographic fatty liver, liver enzyme elevation(including serum ALT>30 IU/L in men and >20 IU/L in women),

or a fatty liver index>35, a value with high specificity to rule out NAFLD in the general population(25).

The study was approved by the local Ethics Committee and conformed to the Helsinki Declaration. Baseline data collected during the period 2004–2005 included: measurements of weight, height, waist circumference, and blood pressure; dietary and physical activity record, routine biochemistry, upper abdomen ultrasound(detailed below).

Frozen serum samples collected from all participants at entry were stored at -80°C for genetic and biochemical analyses. From June 2014 to June 2017, participants in the baseline survey were submitted to follow-up evaluation: all anthropometric, dietary and physical activity records, adbomen ultrasound and biochemical analyses were repeated at the end of follow-up.

Definitions. The diagnosis of NAFLD was based on upper abdomen ultrasound and exclusion of competing causes of steatosis according to current guidelines(detailed in *online Appendix*). Ultrasonographic steatosis was diagnosed using a 3.5 MHz probe according to standardized criteria(26).

Hypertension was defined according to the Eighth Joint National Committee (JNC 8) recommendations (27). Diabetes was defined by a fasting plasma glucose (FPG) \geq 126 mg/dl or 2-h plasma glucose \geq 200mg/dl during an OGTT or by drug treatment for elevated plasma glucose. Obesity was defined by a BMI \geq 30 kg. Insulin resistance was defined by a homeostasis model assessment of insulin resistance(HOMA-IR) index \geq 2, a cut-off closely correlating with clamp measures in Northern Italian subjects and with OGTT-derived indices of insulin sensitivity in NAFLD patients(28). Metabolic syndrome (MS) was defined according to the joint IDF/NHLBI/AHA/IAS/IASO definition(29).

Dietary and physical activity record. Participants were instructed to fill in a 7-day validated and reproducible dietary questionnaire according to the EPIC study(detailed in *online Appendix*), were interviewed about smoking habits and completed the Minnesota-Leisure-Time-Physical-Activity questionnaire(30). The physical activity level was calculated as the product of duration and frequency of each activity (in h/week), weighed by an estimate of the metabolic equivalent of the activity, and summed for activities performed.

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Markers of endothelial dysfunction

AGTR1 activation affects endothelial function (31). Circulating markers of endothelial dysfunction predict subclinical atherosclerosis and early cardiovascular risk in the general population (32) and liver fibrosis in NAFLD(33). We therefore measured circulating soluble endothelial cell adhesion molecules E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cellular adhesion molecule(VCAM-1)(detailed in *online Appendix*).

Circulating adipokines, proinflammatory and pro-/antioxidant markers

AGTR1 activation modulates adipose tissue function and inflammation and oxidative stress, independently of its blood pressure effects(34,35): we therefore measured circulating adipokines adiponectin and resistin and C-reactive protein(CRP)(detailed in *online Appendix*). As oxidative and nitrosative stress play a crucial role in liver and cardio-metabolic disease(36), we also measured plasma nitrotyrosine(NT) and total antioxidant status(TAS)(detailed in *online Appendix*).

Genetic analyses.

Participants were genotyped for AGTR1 rs5186 A>C, PNPLA3 rs738409 C>G and TM6SF2rs58542926 C>T variants with the real-time allele discrimination method, using TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster city, CA). The TaqMan genotyping reaction was run on an 7300HT Fast Real-Time PCR (Applied Biosystem).

We also genotyped our population for *apoE* genotype, a key regulator of lipoprotein metabolism which has been previously linked to the risk of NAFLD(detailed in *online Appendix*).

Impact of AGTR1 rs5186 A>C polymorphism on liver disease, glucose and lipid metabolism.

We next investigated potential mechanisms linking AGTR1 rs5186 A > C variant to liver injury, glucose and lipid metabolism in an independent cross-sectional cohort. Based on previous data, at least 26 subjects per each AGTR1 rs5186 A>C genotype were needed to detect significant differences in

postprandial glucose and lipid metabolism across genotypes(37,38): an independent cohort of 78 biopsy-proven NAFLD patients attending our Turin Hepatological Unit and 78 healthy age-, gender-BMI- and AGTR1 A/C genotype-matched controls, selected among 312 individuals enrolled in a population-based cohort study, underwent a standard OGTT and an oral fat tolerance test(OFTT). A single pathologist (RP) blinded to clinical parameters of the patients read biopsy slides: NAFLD and NASH were diagnosed and staged according to current guidelines(see *online Appendix*). Individuals with diabetes, overt dyslipidemia (fasting serum cholesterol \geq 200 mg/dL or plasma triglyceride \geq 200 mg/dL) or treated with angiotensin receptor blockers(ARBs) were excluded since these conditions may modify the effect of AGTR1 on liver disease, glucose homeostasis, adipokines and lipoprotein metabolism.

Anthropometry.

Body fat % was estimated by the bioelectrical impedance analysis (BIA) method (TBF-202, Tanita, Tokyo, Japan), closely correlating with dual X ray absorption.

Glucose homeostasis.

Minimal Model analysis of plasma glucose, insulin and C-peptide during the OGTT yielded the following parameters of glucose homeostasis(see *online Appendix*): whole-body insulin sensitivity index(OGIS); hepatic and muscle insulin resistance; 2 indexes of β -cell function (the insulinogenic index, IGI, and the CP-genic index, CGI) and 2 integrated indexes of β -cell function, i.e. disposition index (DI) and adaptation index (AI), which relate β -cell insulin secretion to insulin resistance and were previously validated against frequently sampled intravenous glucose tolerance test(FIVGTT) MINIMAL MODEL parameters(39).

Adipose tissue insulin resistance(IR) index was calculated as fasting free fatty acids(FFA) x fasting insulin(37).

Oral fat tolerance test(OFTT).

Western populations spend most of their day in the postprandial state and postprandial lipemia is an emerging CVD risk factor(40). A fat load can induce acute, RAS-mediated endothelial dysfunction and pro-inflammatory activation(41). Growing evidence suggests lipoprotein metabolism and AGTR1

mutually influence each other: circulating apoB-containing lipoproteins upregulate AGTR1 activity(42), while experimental AGTR1 activation stimulates *de novo* lipogenesis, cholesterol synthesis and uptake and intestinal cholesterol absorption((43,44,45).

Hence, biopsy-proven NAFLD patients and controls underwent an OFTT, and the following parameters were measured(detailed in *online Appendix*):

1)plasma lipids and lipoproteins: total cholesterol(Chol), triglyceride(Tg), FFA, HDL-C were measured by automated enzymatic methods. Triglyceride-rich lipoproteins(TRLPs) were isolated through preparative ultracentrifugation, and their apoB48 content was measured(detailed in *online Appendix*).

2)oxidative stress markers: oxidized LDLs(oxLDLs) were measured with a solid phase two-site enzyme immunoassay and plasma Total Antioxidant Status(TAS) was measured as detailed in *online Appendix*.

3)adipokines and chemokines: the adipokines adiponectin and resistin are key players in nutritionally induced liver and cardiovascular inflammation, and the chemokine monocyte chemoattractant protein-1(MCP-1) is central for pro-inflammatory cell recruitment and for profibrogenic progression of CVD and of liver disease in NASH(6).

AGTR1 activation has been linked to adipokine and MCP-1 dysregulation in obesity-related NAFLD and hypertension(46). Hence, circulating adiponectin, resistin and MCP-1 were measured a solid-phase sandwich ELISA(R&D Systems Europe, Abingdon, UK).

4)Nuclear Factor(NF)-κB activation in mononuclear cells(MNCs). The pro-inflammatory transcription factor NF-κB is a master regulator of nutritionally mediated inflammation(47) and NF- κ B activation in mononuclear cells(MNCs) is central for lipid-induced cardiovascular inflammation, endothelial dysfunction and NASH development(6,41). As NF- κ B is a direct downstream target of AGTR1(46,48), we measured NF- κ B activation in circulating MNCs in response to fat ingestion. Briefly, blood samples were collected in sodium EDTA, MNCs were isolated by gradient centrifugation and total cell lysates and nuclear extracts were prepared from freshly isolated MNCs. NF- κ B DNA binding activity was measured with the NF-kBp50/p65 transcription factor enzyme-linked immunosorbent assay(ELISA) kit (Cayman Chemical, Ann Arbor MI, USA)(detailed in *online*

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Appendix). The method separately detects and quantitates p65 and p50 in the nuclear and cytoplasmic fractions: specific NF- κ B activation is demonstrated by translocation of NF- κ B protein from the cytoplasm to nucleus as measured using ELISA. The nuclear NF- κ B(p65) and NF- κ B(p50) DNA-binding activity were quantitated and expressed as nuclear-to-cytoplasm NF- κ B(p65) ratio and nuclear-to-cytoplasm NF- κ B(p50) ratio.

5)Circulating calprotectin. Calprotectin (S100A8/A9) is a heterodimer complex of the two calciumbinding proteins S100A8 and S100A9, which is secreted by activated neutrophils in response to proinflammatory stimuli(49). Calprotectin has direct pro-inflammatory and pro-apoptotic activities and has been recently proposed to initiate hypertensive angiotensin II-induced cardiovascular inflammation and remodeling(50) and liver inflammation in diet-induced NASH models(51), but metabolic triggers of calprotectin secretion in these conditions are unclear. Hypothesizing fat ingestion acutely enhances calprotectin secretion, we measured plasma calprotectin(detailed in *online appendix*).

6)**Markers of hepatocyte apoptosis.** Plasma cytokeratin-18(CK-18) fragments, an hepatocyte-specific marker of apoptosis and hepatic necro-inflammation in NASH(2), were measured by M30-Apoptosense ELISA kit.

Statistical analysis

Participants with and without follow-up visits were compared to determine the appropriateness of an analysis based on participants with complete data only.

Hardy–Weinberg equilibrium was assessed using χ^2 test. Comparisons of genotype frequencies between NAFLD developers and non-NAFLD developers were performed using 2 x 2 contingency tables with χ^2 analysis.

Subjects with and without NAFLD at the end of the follow-up were compared for baseline values and for changes during follow-up. Normality was evaluated by Shapiro-Wilk test and non-normal values were log-transformed for regression analysis.

Fisher or chi-square test were used to compare categorical variables, as appropriate.

Differences across groups were analyzed by ANOVA and then by Bonferroni correction, when variables were normally distributed; otherwise the Kruskal-Wallis test, followed by the post hoc Dunn test, was used to compare nonparametric variables.

To adjust for multiple comparison testing, the Benjamini-Hochberg False Discovery Rate correction was applied to raw p-values in all comparisons; significance was set at an adjusted p-value threshold of 0.05.

Analysis of lifestyle, anthropometric and metabolic parameters and of genetic polymorphisms was made using Spearman correlation test. Genetic polymorphisms were modelled as an additive effect, that is, quantitative predictor variables reflecting the number of risk alleles(0, 1, or 2).

Based on results of univariate analysis, a logistic regression model was used to identify independent predictors of incident NAFLD, and of incident hypertension at the end of follow-up. Variables were selected from parameters which differed between NAFLD developers and non-NAFLD developers at baseline and/or during follow-up.

In the cross-sectional cohort, the area under the curve (AUC) and incremental AUC(IAUC) of parameters measured during the OGTT and the OFTT in NAFLD patients and controls were computed by the trapezoid method. In this cohort, univariate and subsequent multivariate logistic regression analysis were used to identify predictors of glucose and lipid homeostasis parameters and of liver histology in NAFLD patients.

Quartiles of continuous variables were considered in all analyses. Data were expressed as mean±SEM. (STATISTICA software, 5.1, Statsoft Italia, Padua, Italy).

Results

Follow-up cohort: baseline and end-of-follow-up characteristics .

After a mean follow-up period of 9.8 ± 0.2 years, data on 314 participants were available for examination (**Table 1**). The 21(6.7%) individuals lost at follow-up did not significantly differ in baseline anthropometric, lifestyle, clinical or biochemical feature from the other participants included in the analysis. At the end of follow-up, 81(25%) subjects developed NAFLD. NAFLD patients had significantly higher BMI, blood pressure levels, C-reactive protein, markers of endothelial

dysfunction, HOMA-IR, and resistin and lower plasma adiponectin and TAS levels than non-NAFLD developers. NAFLD patients had also a significantly higher incidence of hypertension and of increased Fasting Plasma Glycose (FPG) levels than non-NAFLD developers (**Table 1**).

AGTR1 AA carriers were 49% in non-NAFLD developers vs. 27% in NAFLD developers (p=0.0007), heterozygous AC carriers were 42% in non-NAFLD developers vs. 46% in NAFLD developers (p=0.311) and homozygous CC carriers were 9% in non-NAFLD developers vs. 27% in NAFLD developers(p=0.0001)(**Table 1**). *PNPLA3* G-allele carriers were also more frequent in NAFLD developers than in non-NAFLD developers.

AGTR1 A>C genotype frequencies were in Hardy-Weinberg equilibrium and the minor C-allele frequency(34%) were consistent with published reports in Caucasians(13-16, 18).

At baseline, there was no significant difference in any other genetic, anthropometric, lifestyle, clinical or biochemical feature between NAFLD developers and non-NAFLD developers or among AGTR1 A>C gemotypes (**Table 1**, *Online Appendix Table 1*). There was also no significant difference in physical activity or dietary habits between NAFLD developers and non-NAFLD developers throughout the study (**Table 1**).

Follow-up cohort: impact of *AGTR1 A>C* variant on incidence and severity of NAFLD and on incident hypertension

The presence of *AGTR1* C allele (AC/CC carriers vs. AA carriers) increased the risk of incident NAFLD and hypertension at the end of follow-up(**Table 2**).

Cross-sectional cohort: subject characteristics

Characteristics of NAFLD patients and controls in the cross-sectional cohort are reported in Table 3.

NAFLD patients had NASH in 53% of cases and advanced (Stage 3) fibrosis in 23% of cases. Cirrhotic changes were absent. Mean NAFLD activity score (NAS) in the overall NAFLD cohort was 4.8±0.9. Details on each histological feature are provided in *online Appendix*.

AGTR1 C allele was associated with higher blood pressure, HOMA-IR and circulating markers of oxidative stress, inflammation and endothelial dysfunction within patients and controls, and with more severe liver histology in NAFLD patients(**Table 3**).

Cross-sectional cohort: impact of AGTR1 A>C variant on glucose homeostasis

NAFLD patients as a group had more severe whole-body and tissue insulin resistance than controls (**Table 4**). In both NAFLD patients and controls, the presence of *AGTR1 C* allele was dose-dependently associated with higher whole-body and tissue(hepatic, adipose tissue, muscle) insulin resistance(**Table 4**), while there was no difference across genotypes in parameters related to pancreatic β -cell function (not reported).

On multiple logistic regression analysis, AGTR1 A > C SNP independently predicted whole-body, hepatc and adipose insulin resistance(**Table 5**).

Cross-sectional cohort: impact of AGTR1 A>C variant on oral fat load parameters

1) plasma lipids, lipoproteins and oxidative stress markers

NAFLD patients as a group had a higher postprandial triglyceride and VLDL-Cholesterol levels, and increased intestinal VLDL particles, higher oxLDL and FFA responses and lower postprandial HDL-C and TAS levels than healthy controls (**Table 6**).

LDL-C levels did not significantly change throughout the OFTT.

AGTR1 A>C variant independently predicted postprandial triglyceride, VLDL-Ch and oxLDL responses(**Table 5**).

2)adipokines and chemokines. Despite comparable fasting values, AGTR1 C allele was dose-

dependently associated with lower adiponectin levels and higher resistin and MCP-1 in response to fat ingestion(**Table 6**).

AGTR1 A>C variant predicted postprandial adipokine and MCP-1 responses(**Table 5**).

3)NF-κB activation in mononuclear cells (MNCs), circulating calprotectin and CK-18 fragment levels

Compared with *AGTR1 A* allele, the presence of *AGTR1 C* allele was associated with a higher postprandial NF- κ B activation in MNCs, assessed as both nuclear-to- cytoplasm NF- κ B(p65) and NF-

 κ B(p50) ratio and as nuclear NF- κ B(p65) and nuclear NF- κ B(p50) %DNA binding activity [for simplicity, only NF- κ B(p65) nuclear-to- cytoplasm ratio are reported in **Table 6**]. *AGTR1 C*-allele was also associated with higher circulating calprotectin, a marker of neutrophil activation, and CK-18 fragments, a marker of hepatocyte apoptosis(**Table 6**). *AGTR1 A*>*C* variant independently predicted postprandial NF- κ B activation, calprotectin and CK-18 levels on multiple logistic regression analysis(**Table 5**).

Cross-sectional cohort: predictors of liver histology (Table 5).

The presence of **NASH** was predicted by postprandial calprotetin and NF- κ B activation in MNCs. The presence of **advanced** (**stage 3**) **fibrosis** was predicted by . *AGTR1 A*>*C* variant, by postprandial VLDL-Cholesterol response and by NF- κ B activation in MNCs(**Table 5**).

Cross-sectional cohort: effect of AGTR1 A>C variant on liver histology, endothelial dysfunction and metabolic parameters in non-hypertensive NAFLD patients

The effects of AGTR1 A > C variant on liver histology, endothelial dysfunction, insulin sensitivity and postprandial lipemia in the subgroup of non-hypertensive NAFLD patients (n=43) were similar to those in the overall cohort of NAFLD patients(**Figure 1**)

Discussion

The main findings of our study are the following:

- In non-obese, normotensive insulin sensitive normolipidemic individuals, *AGTR1 A>C* variant predicted incident NAFLD and hypertension at the end of follow-up.
- AGTR1 A>C variant affects postprandial lipid metabolism, fat-induced pro-inflammatory leukocyte activation, and adipose tissue dysfunction, which may contribute to liver injury and endothelial dysfunction, leading to NASH and hypertension

NAFLD was replicated in an independent cohort of biopsy-proven NAFLD patients and controls Growing experimental evidence places inappropriate AGTR1 activation at the core of a network of signaling pathways mediating organ injury and metabolic disturbances in hypertension and NAFLD, including proinflammatory and profibrotic cytokine and MCP-1 chemokine release, enhanced NFkB-mediated proinflammatory cell activation , and endothelial dysfunction(6,12,34,46). We therefore investigated the impact of the common gain-of-function *AGTR1 rs5186 A>C* variant on the development and severity of NAFLD and on NAFLD-associated hypertension: we found AGTR1 A>C variant predicted the risk and severity of NAFLD and incident hypertension in a cohort of healthy, nononbese, normotensive nondiabetic insulin sensitive individuals.

A number of mechanisms may mediate hepatic and vascular effects of AGTR1 activation (**Figure 2**). Our data suggest enhanced acute fat-induced proinflammatory response is a central mediator of the effects of *AGTR1 A>C* variant on liver injury and endothelial dysfunction, as increased NF- κ B actvation in MNCs and calprotectin response independently predicted liver histology, hepatocyte apoptosis and endothelial dysfunction(**Table 5**). Postprandial fat-induced activation of NF- κ B is of particular pathogenic relevance, as NF- κ B is a key transciption factor mediating nutritionally-induced release of pro-inflammatory and pro-fibrogenic adipokines and chemokines(47),which is believed to play a pivotal role in metabolic inflammation, adipose tissue dysfunction and liver injury in NASH(6,48).

Consistent with experimental evidence indicating NF- κ B is a direct downstream target of AGTR1(47,48), we found MNC NF- κ B activation in response to fat ingestion is modulated by *AGTR1 A>C* variant (**Table 5-6**)

Intriguingly, AGTR1 A > C variant also modulated postprandial fat-induced increase in circulating calprotectin, a neutrophil-derived pro-inflammatory, chemotactic and pro-apoptotic heterodimeric protein whose key role in initiating cardiovascular and metabolic liver inflammation is being increasingly recognized(46,47).

Collectively, these data indicate a pervasive role of AGTR1 in modulating the inflammatory response to an acute, repetitive stimulus like fat ingestion and suggest *AGTR1 A>C variant* may be

a key modifier of genetic susceptibility to dietary fat lipotoxicity, configuring the adaptive failure to dietary fat which is observed in nutritionally induced models of NASH(52, 53).

Beside circulating leukocytes, adipose tissue has a high degree of expression of ATIIR in humans(22) and adipose tissue function seems also to be modulated by AGTR1 A > C variant: C alele carriers showed in fact adipose insulin resistnce, which enhances lipotoxic FFA release to the liver, and pro-/anti-inflammatory adipokine imbalance, with a prevalent pro-inflammatory adipokine and chemokine response over the anti-inflammatory adiponectin (**Table 5-6**).

Finally, AGTR1 A > C variant seems also to directly induce dysregulated postprandial lipoprotein metabolism, with the C allele predisposing to postprandial triglyceride- and cholesterol-rich VLDL lipoprotein accumulation (**Table 6, Figure 2**). This is consistent with recent experimental data linking RAS activation to upregulation of intestinal cholesterol absorption (44,45), *de novo* lipogenesis and cholesterol synthesis and uptake from the circulation(43,54).

In summary, our study expands our knowledge on the role of AGTR1 in liver and CVD disease in NASH: beside a direct modulation of hepatic stellate cell activity and fibrogenesis(12,13,14), inappropriate ATIIR1 activation predisposes to NASH and hypertension through enhanced intestinal cholesterol absorption and cholesterol overload, inflammatory cell activation, and adipose tissue dysfunction (**Figure 2**).

If confirmed by larger studies, these findings may have screening and therapeutic implications. First, AGTR1 A > C variant may help stratify liver-related and cardio-metabolic risk of NAFLD patients: specifically, it will be important to evaluate if genotyping NAFLD patients for this AGTR1 variant may help select those individuals who may benefit most from ARB treatment, as it has been shown for hypertensive patients with metabolic syndrome(20,21).

Second, as circulating lipoproteins upregulate AGTR1 activity(42), postprandial lipoprotein accumulation may synergize with genetically induced AGTR1 activation in C allele carriers to promote tissue lipid accumulation, inflammation and organ injury(39-43). Hence, it will be important to evaluate of combining ARBs with drugs targeting postprandial lipemia, like statins and selective peroxisome proliferator-activated receptor α modulators (SPPARM α), even in the absence of fasting

hyperlipidemia, may offer synergistic benefits on liver disease and cardio-metabolic profile in NASH(6, 14,41,50,55,56).

Third, the small subgroup of nonhypertensive NAFLD patients showed similar liver disease severity and cardio-metabolic profile as the whole NAFLD cohort (*Figure 1*). These findings may provide the basis for evaluating the benefits of ARB treatment in NAFLD *AGTR1* C allele carriers with prehypertension (defined as SBP/DBP 120-139/80-89 mmHg), as recent literature highlights the need to consider lower blood-pressure goals in antihypertensive treatment of selected patients with an elevated cardiovascular risk profile(57).

Our study has strengths and limitations: strengths are the careful selection, the thorough characterization of participants and the histological characterization of NAFLD, limitations are the relatively small sample size, and the ultrasonographic method to screen for NAFLD, which may have missed some cases of milder steatosis. However, we used standardized criteria with significantly improved diagnostic accuracy for detecting even minor degrees of steatosis(26), and the combination of ultrasonography with recently proposed lower ALT cut-off values(25) and fatty liver index should have enhanced individuation of these cases.

Our findings may provide the basis for large trials evaluating the benefits of ARBs, alone or in synergy with drugs targeting lipid metabolism or NF-kB activation(⁵⁸), on liver disease and cardiometabolic risk in NASH, as well as the interaction of these pharmacological treatments with with ATRII genotype in these patients.

FIGURE LEGENDS

Figure 1

Cross-sectional cohort: steatosis severity (% hepatocytes involved)(panel A), NAFLD Activity score (NAS, panel B), Fibrosis stage (panel C), plasma E-selectin(panel D), OGIS(panel E) and postprandial Tg(panel F) in non-hypertensive patients with biopsy-proven NAFLD, grouped according to AGTR1 A/C genotype (n=43). Data are expressed as mean±SEM

Figure 2

Proposed mechanisms linking AGTR1 activation to NASH and hypertension, based on the results of our study and on literature findings.

In the intestine, inappropriate AGTR1 activation enhances intestinal absorption of dietary cholesterol, which accumulates in the vessel walls and in the liver contributing to endothelial dysfunction and liver injury.

AGTR1 activation triggers fat-induced activation of CIRCULATING polymorphonuclear neutrophils (PMNs) and mononuclear cells(MNCs), which accumulate in the vessel wall, liver and adipose tissue contributing to tissue inflammation and organ dysfunction.

AGTR1 is also prominently expressed in adipocytes and its inappropriate activation promoted adipose tissue dysfunction, which includes resistance to the antilipolytic action of insulin, leading to lipotoxic free fatty acid release, and to pro-/anti-inflammatory adipokine imbalance.

Finally, inappropriate AGTR1 activation on hepatic stellate cells(HSC) triggers HSC activation and hepatic fibrogenesis

Table 1 Baseline, end-of-follow-up and changes during follow-up in main clinical and biochemicalfeatures of the follow-up cohort(n=314), grouped according to the development or not of NAFLD atthe end of follow-up.

	Base	eline	Р	End-of-fo	llow-up	Р	Changes during	g follow-up	Р
Parameter	Non-	NAFLD		Non-NAFLD	NAFLD		Non-NAFLD	NAFLD	
	NAFLD	developers		developers	developers		developers	developers	
	developer	(n=81)		(n=233)	(n=81)		(n=233)	(n=81)	
	s								
	(n=233)								
Age(yr)	49±1	49±1	0.998	59±1	59±1	0.712	10±1	10±1	0.912
Sex(%M)	57	63	0.613	-	-	-	-	-	-
BMI(kg/m ²)	25.8±0.2	25.9±0.3	0.815	27.1±0.2	27.9±0.3	0.01	1.3±0.2	2.1±0.2	0.010
Waist (cm)	90±1	91±2	0.728	95±2	97±2	0.328	5±1	6±1	0.579
Sys BP	125±2	121±2	0.729	133±2	141±2	0.027	8±1	20±2	0.0007
(mmHg)									
Dia BP	82±1	81±1	0.872	85±1	91±2	0.003	3±1	9±1	0.009
(mmHg)									
Hypertension	0	0	0.999	81(35%)	43(53%)	0.003	89(38%)	47(58%)	0.003
(%)*									
AGTR1 A/C									
n (%)				-	-	-	-	-	-
AA	115(49)	22(27)	0.0007						
AC	98(42)	37(46)	0.311						
CC	20(9)	22(27)	0.0001						
PNPLA3 C/G				-	-	-	-	-	-
n(%)									
C/C	134(58)	20(25)	0.001						
C/G	68(29)	38(47)	0.091						
G/G	31(13)	23(28)	0.023						
TM6SF2 C/T									
n(%)					-	-	-	-	-
CC	200(87)	64(79)	0.792						
СТ	30(12)	12(15)	0.827						
TT	3(1)	5(6)	0.318						
ApoE n(%)									
2-3	46(19)	18(22)	0.895	-	-	-	-	-	-
3-3	141(61)	46(57)	0.724						
3-4	46(20)	17(21)	0.812						
Education:		<i></i>	0.022						
Primary	65	64	0.832	-	-	-	-	-	-
school	25	22	0.011						
Secondary	25	22	0.811						
school									

University	10	14	0.793						
Smoking									
status:									
never(%)	65	63	0.712	67	65	0.812	+2	+2	0.812
former(%)	18	19	0.710	15	16	0.893		-3	0.872
E-selectin	22.1±3.0	21.6±3.1	0.825	25.2±3.5	39.5±3.1	0.0005	3.0±1.3	17.9±3.0	0.0001
(ng/mL)									
ICAM-1	200.1±8.1	198.8±9.1	0.815	221.1±7.9	269.3±7.9	0.0001	20.9±8.1	71.6±10.2	0.0001
(ng/mL)									
VCAM-1	432.6±19.5	441.9±16.4	0.713	538.9±24.1	621.3±32.	0.009	106.2±8.4	178.7±8.6	0.002
(ng/mL)					1				
Resistin(ng/m	3.4±0.3	3.5±0.3	0814	3.7±0.3	4.9±0.4	0.092	0.3±0.3	1.4±0.3	0.031
L)									
Adiponectin	7814±218	7582±238	0.718	7101±197	5738±182	0.009	-799±97	-1845±216	0.0001
(ng/mL)									
Glucose	92±3	93±3	0.896	96±3	101±3	0.812	4 <u>±</u> 2	7±2	0.191
(mg/dL)									
Insulin	7.4±0.5	7.5±0.6	0.812	9.2±0.9	15.9±1.2	0.001	1.8±0.4	7.8±0.7	0.0005
(µU/mL)									
HOMA-IR	1.5±0.4	1.5±0.4	0.916	2.0±0.3	4.2±0.3	0.008	0.5±0.3	2.7±0.3	0.0001
(mmol/l									
x μU/ml)									
Tg(mg/dL)	114±10	116±9	0.788	118±9	125±11	0.281	4 <u>±</u> 6	10±9	0.519
Total	185±7	190±7	0.799	201±9	209±6	0.114	15±4	19±4	0.392
C(mg/dL)									
LDL-C	100±8	100±7	0.682	129±6	138±5	0.319	28±5	38±8	0.319
(mg/dL)									
HDL-C	61±2	61±2	0.811	53±1	51±2	0.716	-8±1	-10±2	0.219
(mg/dL)									
AST(IU/L)	16±1	16±1	0.999	19±1	41±3	0.0001	1±1	23±2	0.0002
ALT(IU/L)	18±1	18±1	0.914	22±2	81±5	0.0001	2±1	62±2	0.0001
GGT(IU/L)	20±4	20±4	0.925	22±4	89±4	0.0001	2±1	60±2	0.0001
T2DM(%)	0	0	0.999	7(3)	11(14)	0.0009	-	-	-
Met Sy(%)#	0	0	0.999	23(10)	22(27)	0.291	-	-	-

The P values value refer to comparison between groups at baseline, at the end of follow-up and to comparison in changes during the follow-up, respectively(statistically significant p-values are written in bold chatacters). Data are expressed as mean±SEM.

Subjects with and without NAFLD at the end of the follow-up were compared for baseline values and for changes during follow-up.

Abbreviations: BP: blood pressure; C: cholesterol; CRP: C-reactive protein; Dia: diastolic; HOMA-

IR: homeostasis model assessment of insulin resistance; ICAM: intercellular adhesion molecule; PNPLA3: patatin-like phospholipase domain-containing 3; Sys: systolic; T2DM: type 2 diabetes mellitus; Tg: triglycerides;

*Hypertension was diagnosed according to the Eighth Joint National Committee (JNC 8) recommendations(22).

Metabolic syndrome (Met Sy) was defined according to the joint statement of American Heart association (AHA), International Diabetes Federation (IDF) and National Heart Lung and Blood Institute (NHLBI)(24). **Table 2.** Multiple logistic regression analysis for predictors of incident NAFLD, NASH, advanced fibrosis, hypertension and of increased levels of endothelial dysfunction markers (highest E-selectin, ICAM-1 and VCAM-1) quartiles in the follow-up cohort (n=314).

Incident NAFLD at end of follow-up							
Parameter	OR (95%CI)	Р					
AGTR1 SNP(AC/CC vs. AA)	1.67 (1.26-2.21)	0.002					
PNPLA3(CG/GG vs. CC)	1.46(1.13-1.97)	0.013					
Changes in BMI	1.39(1.07-1.81)	0.021					
Changes in HOMA-IR	1.38(0.71-2.11)	0.639					
Changes in E-selectin	1.27(0.71-1.64)	0.729					
Changes in ICAM-1	1.01(0.55-1.59)	0.739					
Changes in C-reactive protein	1.25(0.75-1.78)	0.812					
Changes in fasting serum adiponectin	0.72(0.25-1.24)	0.218					
Changes in fasting serum resistin	1.29(0.74-1.79)	0.836					
Incident hyperten	sion at end of follow-up						
AGTR1 SNP(AC/CC vs. AA)	1.49(1.12-2.63)	0.015					
Changes in BMI	1.21(0.64-1.71)	0.819					
Changes in E-selectin	1.48(1.09-2.21)	0.031					
Changes in ICAM-1	1.45(1.11-1.84)	0.030					
Changes in C-reactive protein	1.21(0.80-2.18)	0.479					
Changes in fasting serum adiponectin	0.69(0.22-1.23)	0.347					
Changes in HOMA-IR	1.18(0.31-2.07)	0.813					

Odds Ratios (OR) and 95% Confidence Intervals are reported. Quartiles of continuous variables were considered in logistic regression analysis. For SNPs, the risk is intended per at risk allele.

Abbreviations: HOMA-IR: homeostasis model assessment of insulin resistance; ICAM: intercellular adhesion molecule; VCAM: vascular cellular adhesion molecule; PNPLA3: patatin-like phospholipase domain-containing 3;

Table 3. Main clinical, biochemical and histological parameters of NAFLD patients and controls

		Controls			NAFLD	
	AGTR1 AA (n=26)	AGTR1 AC (n=26)	AGTR1 CC (n=26)	AGTR1 AA (n=26)	AGTR1 AC (n=26)	AGTR1 CC (n=26)
Age(yr)	56±2	55±2	55±2	56±2	55±2	55±2
Sex (%M)	60	59	56	60	59	56
Smokers (%)	22	21	21	20	24	21
BMI (kg/m ²)	28.1±0.4	28.5±0.4	28.3±0.3	28.1±0.5	28.5±0.5	28.3±0.4
Waist (cm)	95±3	96±3	95±3	96±3	96±3	95±3
Sys BP (mmHg)	125±6	135±2	139±2	127±2	135±3	142±2†
Dia BP (mmHg)	78±1	82±1	86±1	82±1	86±1	90±1†
Hypertension(%) ¹¹	31	40	51†	39	44	55†
Glucose (mg/dL)	87±3	90±3	93±3†	97±3	100±3	105±3†
Insulin (µU/mL)	6.1±0.6*	9.3±1.1	11.7±0.6†	12.3±2.0*	21.1±2.1	26.2±2.1†
HOMA-IR	1.3±0.3*	2.0±0.7*	2.9±1.0*	3.5±0.6*	5.4±0.9*	6.3±1.1*
Total C (mg/dL)	178±5	181±5	179±6	180±6	178±9	118±8
LDL-C (mg/dL)	107±7	105±8	110±9	110±7	111±8	119±6
AST (IU/L)	16±2	19±2	20±2	32±2*	41±3*	52±3*
ALT (IU/L)	17±2	20±2	19±2	51±4*	75±3*	82±3*
PNPLA3 (%) CC CG/GG	47 53	51 49	50 50	39 61	40 60	37 63
TM6SF2 (%) CC	89	90	88	87	86	85
CT/TT	11	10	12	13	14	15
ApoE (%) 2-3 3-3 3-4 E- selectin (ng/mL)	16 63 21 15.1±3.4*	15 66 19 22.8±2.1*	19 62 19 34.3±1.7	15 66 19 26.1±2.6*	13 66 21 35.2±2.1*	16 65 19 49.2±2.1*
ICAM-1 (ng/mL)	163.4±4.8*	192.2±5.4*	244.2±5.3*	199.1±5.1*	265.3±6.2*	306.5±7.4*
VCAM-1 (ng/mL)	418.2±15.2*	512.9±16.3 *	594.6±18.9*	599.1±16.4*	622.5±17.9*	665.9±19.5 *
T2DM(%)	0	0	0	0	0	0
Met sy(%)#	21	23	28	42§	43§	46§
NAS	-	-	-	1.9±0.2**	3.7±0.2**	5.9±0.2**

Fibrosis stage	-	-	-	0.6±0.2**	1.3±0.2**†	2.5±0.2**
Patients with severe steatosis n (%)				4(15) **	9(35)*	++ 17(65)**
Patients with NASH n (%)	-	-	-	8(53) **	15(57)**	23(88)* *
Patients with advanced fibrosis n (%)	-	-	-	3(12) **	7(27) **	15(58) **
Patients with cirrhosis n (%)				0(0)	0(0)	0(0)

Data are expressed as mean±SEM. Statistically significant P values are written in bold characters.

¹Hypertension was diagnosed according to the Eighth Joint National Committee (JNC 8)

recommendations(32): a SBP \geq 140 and/or DBP \geq 90 mmHg (values reported are the mean of the two determinations) *or* on antihypertensive therapy.

[†] values indicates percent hypertensive patients (as diagnosed according to JNC 8) on antihypertensive therapy

#Metabolic syndrome (Met Sy) was defined according to the joint statement of American Heart association (AHA), International Diabetes Federation (IDF) and National Heart Lung and Blood Institute (NHLBI)(34)

Abbreviations: BP: blood pressure; C: cholesterol; CRP: C-reactive protein; Dia: diastolic; HOMA-IR: homeostasis model assessment of insulin resistance; ICAM: intercellular adhesion molecule; PNPLA3: patatin-like phospholipase domain-containing 3; Sys: systolic; T2DM: type 2 diabetes mellitus; Tg: triglycerides;

* p<0.05 vs. subjects with the other AGTR1 A/C genotypes with the same condition (NAFLD or controls)

+ p<0.05 vs. AA genotype group with the same condition (NAFLD or controls)

[‡] p<0.05 vs. subjects with AC genotype group with the same condition (NAFLD or controls)

** p<0.01 vs. subjects with the other AGTR1 A/C genotypes with the same condition (NAFLD or controls)

⁺⁺ p<0.01 vs. subjects with AA genotype with the same condition (NAFLD or controls)

§ p<0.05 vs. all control groups

Data are presented as mean \pm SEM, unless otherwise specified.

Abbreviations. BP: blood pressure; total C: total cholesterol; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; NAS: NAFLD Activity Score (sum of steatosis, lobular inflammation and ballooning scores); Tg: triglyceride; IGR: impaired glucose regulation; IFG: impaired fasting glycaemia; IGT: impaired glucose tolerance

Severe steatosis was defined as involving >66% hepatocytes; NASH was defined according to Brunt definition; Advanced steatosis was defined as stage 3 fibrosis(no patient had cirrhosis on liver biopsy).

Table 4. Glucose homeostasis parameters of biopsy-proven NAFLD patients and controls, grouped according to AGTR1 A>C genotype in the cross-sectional cohort (n=156).

	Controls			NAFLD			
	AGTR1	AGTR1	AGTR1	AGTR1	AGTR1	AGTR1	
	AA	AC	CC	AA	AC	CC	
	(n=26)	(n=26)	(n=26)	(n=26)	(n=26)	(n=26)	
OGIS	476.3±	453.1±	421.1±	387.8±10.1	354.3±8.3	328.7±8.2	
(ml · min ⁻¹ · m ⁻²)	19.2**	13.5**	16.4**	**	**	**	
Hepatic insulin resistance (g/dL _{glucose} · μU/mL _{Ins} ·min ⁻²)	2046± 171**	3696±196 **	4287± 97**	5196±113**	5785±124**	6562±152 **	
Adipose IR (mmol/L * µU/mL)	2.1±0.3*	4.6±0.8*	6.7±1.2**	8.6±1.7**	13.5±2.2**	19.6±2.3**	
Muscle IS	0.037±0.00	0.028±	0.022±	0.020±0.001	0.013±0.001*	0.008±0.001	
	1*	0.001*	0.001*	**	*	**	

Data are presented as mean \pm SEM.

* p<0.05 vs. subjects with the other AGTR1 genotypes with the same condition (NAFLD or controls)
** p<0.001 vs. subjects with the other AGTR1 genotypes with the same condition (NAFLD or controls)

Table 5. Multiple logistic regression analysis. independent predictors of liver histology (panel A),

endothelial dysfunction (panel B), glucose homeostasis (panel C), and oral fat tolerance test (panel D) in the cross-sectional cohort.

A)Liver histology*									
NASH									
IAUC calprotectin	1.73(1.31-2.15)	0.001							
IAUC nuclear NF-κB	2.37(1.71-3.06)	0.001							
Adva	Advanced (Stage 3) fibrosis								
AGTR1 A/C (AC/CC vs. AA)	1.46 (1.19-1.74)	0.007							
IAUC nuclear NF-кВ	1.71(1.26-2.16)	0.009							
IAUC VLDL-Cholesterol	1.49(1.18-1.80)	0.012							
B) End	lothelial dysfunction								
	E-selectin								
IAUC calprotectin	1.51(1.18-1.82)	0.011							
IAUC oxLDL	1.62(1.11-2.13)	0.003							
	ICAM-1	I							
IAUC nuclear NF-κB	1.79(1.31-2.20)	0.002							
IAUC VLDL-Cholesterol	1.52(1.12-2.28)	0.009							
C) G	lucose homeostasis								
	OGIS								
Independent variable	OR(95% CI)	Р							
AGTR1 A/C (AC/CC vs. AA)	0.57(0.25-0.79)	0.001							
IAUC adiponectin	1.48 (1.19- 2.20)	0.009							
Hepatic insulin resistance									
AGTR1 A/C (AC/CC vs. AA)	1.75 (1.29-2.25)	0.003							
IAUC adiponectin	0.48 (0.36-0.61)	0.001							
	Adipose IR								
AGTR1 A/C (AC/CC vs. AA)	1.82 (1.342.31)	0.0009							

IAUCcalprotectin	2.01(1.56-2.47)	0.007							
Muscle IS									
IAUC adiponectin	1.98 (1.60-2.56)	0.004							
D) Oral fat tolerance									
IAUC VLDL-Ch									
AGTR1 A/C (AC/CC vs. AA)	1.57(1.152.01)	0.011							
	IAUC oxLDL								
AGTR1 A/C (AC/CC vs. AA)	1.51 (1.15-1.86)	0.011							
IAUC triglyceride	2.01(1.522.54)	0.0009							
L	AUC adiponectin								
AGTR1 A/C (AC/CC vs. AA)	0.48(0.19-0.81)	0.009							
IAUC resistin									
AGTR1 A/C (AC/CC vs. AA)	1.89(1.35-2.56)	0.002							
	IAUC MCP-1	•							
IAUC calprotectin	1.44(1.13-1.78)	0.019							
IAUC nuclear NF-κB	2.12(1.79-2.41)	0.0002							
IAU	JC NF-κB in MNCs								
AGTR1 A/C (AC/CC vs. AA)	0.44 (0.32-0.59)	0.004							
IAUC calprotectin	1.59(1.18-2.02)	0.009							
I	AUC calprotectin								
AGTR1 A/C (AC/CC vs. AA)	2.68(2.19-3.21)	0.0001							
IAUC oxLDL	2.01(1.522.54)	0.0009							
IAU	C CK-18 fragments	•							
Fasting CK-18 fragments	1.49(110-1.96)	0.008							
IAUC nuclear NF-κB	2.21(1.85-2.58)	0.008							
IAUC calprotectin	1.66(1.11-2.21)	0.008							
N		•							

*analysis performed in the 78 biopsy-proven NAFLD patients.

Only parameters significantly associated with outcome variables are shown

Abbreviations: Tg: triglyceride; Glu: glucose; C: cholesterol; MCP:monocyte chemoattractant

protein; MNC: mononuclear cells: NF-κB: nuclear factor-κB

 Table 6. Oral fat load parameters of patients with NAFLD and controls grouped according to AGTR1

 A: Construction in the state of the st

		Controls			NAFLD	
	AGTR1 AA (n=26)	AGTR1 AC (n=26)	AGTR1 CC (n=26)	AGTR1 AA (n=26)	AGTR1 AC (n=26)	AGTR1 CC (n=26)
Fasting Tg (mg/dL)	95±21	108±19	115±20	99±18	103±19	95±16
IAUC Tg (mg/dL x hr)	69±16*	105±19*	181±21*	267±29*	395±30*	608±40 *
Fasting VLDL-apoB48 (mg/dL)	58±26	63±28	71±39	66±41	79±44	83±51
IAUC VLDL-apoB48 (mg/dL x hr)	36±13*	32±21*	58±34*	89±26*§	123±31 *	182±43*
Fasting VLDL-Ch (mg/dL)	26±5	25±5	24±4	29±6	28±9	29±7
IAUC VLDL-Ch	22±5*	58±5*	97±7*	87±16*	141±28*	195±29*
(mg/dL x hr) Fasting FFA (mMol/L)	0.30±0.21	0.40±0.19	0.46±0.21	0.49±0.11§	0.57±0.18	0.65±0.19§
IAUC FFA (mMol/L x hr)	0.96±0.07*	1.11±0.21*	1.97±0.42*	2.28±0.51*	3.15±0.72*	4.76±0.91*
Fasting HDL-C(mg/dL)	56±2	55±2	54±2	50±2	46±2	43±2
IAUC HDL-C(mg/dL x hr)	-2±2	-6±2	-10±2†	-14±2	-18±2	-22±3†
Fasting oxLDL (IU/L)	43±3	42±3	45±3	48±4	54±4	60±5
IAUC oxLDL (IU/L)	7±2**	27±5**	37±6**	117±11**	141±15**	184±17*
Fasting TAS (mmol/L)	0.60±0.03 *	0.56±0.03*	0.47±0.02*	0.47±0.02 *	0.41±0.02 *	0.40±0.03 *
IAUC TAS (mmol/L x hr)	0.77±0.10 **	0.51±0.06 **	0.33±0.09 **	0.11±0.04 **	-0.36±0.08 **	-1.15±0.09 **
Fasting adiponectin (ng/mL)	11032±839	10301±812	19992±842	6828±612 §	7004±571 §	6457 ±412 §
IAUC adiponectin (ng/mL x hr)	26529±912 **	13082±973* *	8251± 712**	5417± 371	-2741± 511**	-9907± 794**
Fasting resistin (ng/mL)	3.46±0.45	3.89±0.48	3.72±0.49	3.70±0.50	3.62±0.48	4.15±0.50
IAUC resistin (ng/mL x hr)	0.51±0.13 *	1.68±0.39 *	3.53±0.41 *	1.45±0.42 **	5.56±0.81 **	9.94±1.02 **
Fasting MCP-1 (pg/mL)	145±21	152±18	159±20	169±21	181±22	191±23

A>C genotype in the cross-sectional cohort (n=156).

IAUC MCP-1 (pg/mL x hr)	92±11*	143±16*	211±17**	162±16*	298±15*	445±17**
Fasting cytokeratin-18 (U.I./L)	92±8	102±10	110±9	190±9*	207±11*	261±17*
IAUC cytokeratin-18 (U.I./L x hr)	68±9*	120±10*	179±14*	221±13#**	335±11 †#**	459±15**
Fasting MNCs nuclear-to-cytoplasm NF-кB ratio	0.91±0.10	1.09±0.21	1.19±0.29	1.02±0.13	1.18±0.39	1.49±0.27
IAUC MNCs nuclear- to-cytoplasm NF-кB ratio	0.410.11**	1.45±0.39**	2.41±0.43**	1.83±0.41**	4.18±1.11**	9.19±1.52**

Dat are presented as mean \pm SEM.

Abbreviations: Tg: triglyceride; C: cholesterol; MCP:monocyte chemoattractant protein; MNCs:

mononuclear cells: NF-κB: nuclear factor-κB

* p<0.05 vs. subjects with the other AGTR1 A/C genotypes with the same condition (NAFLD or controls)

⁺ p<0.05 vs. AA genotype group with the same condition (NAFLD or controls)

[‡] p<0.05 vs. subjects with CC genotype group with the same condition (NAFLD or controls)

** p<0.001 vs. subjects with the other AGTR1 A/C genotypes with the same condition (NASH or controls)

⁺⁺ p<0.001 vs. subjects with AA genotype with the same condition (NAFLD or controls)

p<0.001 vs. subjects with CC genotype within the same group (NAFLD or controls)

Figure 1





Panel B











Panel E







Abbreviations: NAS: NAFLD activity score; OGIS: oral glucose insulin sensitivity index: Tg:

Triglyceride

 \bigstar p <0.01 vs. other genotypes

Figure 2. Proposed mechanisms linking ATIIR1 activation to NASH and hypertension, based on





ONLINE APPENDIX

METHODS

Criteria for excluding competing causes of hepatic steatosis. Criteria for excluding competing causes of fatty liver disease were as follows: a history of alcohol consumption >20 g/d (males) and >10 g/d (females) (assessed by a detailed inquiry of patients and relatives and a validated questionnaire filled in daily for one week by the patients) or positive serum markers of viral hepatitis B-C; exposure to occupational hepatotoxins or drugs known to be steatogenic or hepatotoxic; positive autoimmune or celiac disease markers; abnormal serum α_1 -antitripsin, ceruloplasmin, trasferrin saturation, or thyroid hormones. Mutations in the hemochromatosis genes HFE and TRF2 were detected in patients and controls using multiplex amplification reaction (Nuclear Laser Medicine, Milan, Italy). Liver iron concentration (LIC) and hepatic iron index (HII) were assessed from 2 mg dry weight tissue by atomic absorption spectroscopy.

Genetic analyses. Primers and probes for allelic discrimination analysis of AGTR1 A1166C were as follows:forward primer AGCAAGAGAACATTCCTC, reverse primer CAGCCGTCATCTGTCTA, A1166 probe (FAM-labeled)-5'CTACCAAATGAGCATTAGCT-3' (BLACK HOLE1), C1166 (TEXAS RED labelled)-CTACCAAATGAGCCTTAGCT- 3' (BLACK HOLE2).

Purified DNA (2 μ l) was amplified in a real-time PCR in the iCycler iQ system (BIO-RAD, Hercules, CA). Positive controls, genotyped by direct sequencing, were included in each run, together with a negative control containing no DNA template

Markers of endothelial dysfunction, inflammation and adipokines

Serum E-selectin and ICAM-1 levels were measured by a solid phase ELISA (R&D SYSTEM, Minneapolis, MN, USA). Minimum detectable doses and intra- and inter-assay CVs were, respectively, <0.1 ng/mL, 4.7-5.0 %, 7.4-8.8%, and 0.17-1.26 pg/mL, 2.3-3.6%, 5.5-7.8%,.

Serum CRP levels were determined via a high sensitivity latex agglutination method on HITACHI 911 Analyser (Sentinel Ch., Milan). The kit had a minimum detection of less than 0.05 mg/L, and a measurable concentration range up to 160 mg/L. The intra-assay and inter-assay variation coefficients were, respectively, 0.8–1.3 and 1.0–1.5%.

Serum adiponectin and plasma MCP-1 were measured by a sandwich ELISA (R&D Systems Europe, Abingdon, UK); the kit has a sensitivity of 0.25 pg/ml in a 50-µl sample size and a range of 3.9 to 250 ng/ml. The intra- and interassay CVs were 3.4 and 5.8%, respectively.

Resistin was measured by a biotin-labeled antibody- based sandwich enzyme immunoassay (BioVendor Laboratory Medicine, Brno, Czech Republic). The intra-assay e inter-assay CVs were respectively 2.8–3.4% and 5.5–6.8%.

Pro-/antioxidant markers. Plasma nitrotyrosine (NT) was measured by a commercial ELISA kit product by HyCult Biotechnology (Pantec, Turin, Italy).

Plasma total antioxidant status (TAS) measurement was based on the reduction of Cu++ into Cu+ by the action of all present antioxidants. The amount of Cu+ is evaluating through measuring the complex formed by Cu+ and bathocuproine. This complex has a typical absorption at 490 nm. (ANTOXT Kit by Fujirebio Diagnostics AB Göteborg, Sweden)⁻

Genetic analyses. ApoE genotype was determined by PCR amplification of genomic DNA using specific oligonucleotide primers.

Dietary and physical activity record. Subjects were instructed to fill in a 7-day validated and reproducible dietary questionnaire during an individual training session with a nutritionist; a list of foods was designed, for each item different portion sizes were specified according to the EPIC study(59). The diet record was analyzed using the WinFood database (Medimatica, Teramo, Italy) according to the table of food consumption of the Italian National Institute of Nutrition and Food Composition Database for Epidemiological Study in Italy(60).

Oral glucose tolerance test (OGTT)-derived indexes of glucose homeostasis.

Plasma glucose, insulin and C-peptide were measured at every time-point. The area under the concentration curve (AUC) of glucose, insulin and C-peptide during the OGTT was calculated with the trapezoidal method. Prehepatic insulin delivery was estimated as the suprabasal (Δ) 30-min AUC of C-peptide divided by the 30-min increase in circulating glucose. The hepatic insulin extraction (He), as percentage of secreted hormone, was estimated by [1 – (AUC insulin/AUC C-pep)].

Insulin sensitivity was estimated from a model of glucose clearance, which provides the oral glucose insulin sensitivity (OGIS), an index of whole body insulin sensitivity; muscle and hepatic insulin resistance index were calculated from OGTT as previously proposed and validated against clamp in nondiabetic subjects(1, 2).

The following indexes of β -cell function were also calculated: the insulinogenic index (IGI), computed as the suprabasal serum insulin increment divided by the corresponding plasma glucose increment in the first 30 min (Δ I30/ Δ G30), and the CP-genic index (CGI), computed as Δ C-pep30/ Δ G30, previously validated against measures of β -cell functions derived from frequently-sampled intravenous glucose tolerance test (FIVGTT)(3-5). Two integrated indexes of β -cell function, i.e. disposition index (DI) and adaptation index (AI), were also calculated. DI and AI relate β -cell ability to adapt insulin secretion to changes in insulin sensitivity and represent integrated parameters of β -cell function. These indexes also accurately predicted future T2DM in the general population(6).

Oral fat load test: meal composition

Participants were encouraged to avoid strenuous physical efforts and to follow their usual diet during the 24 h preceding the test. The fat load consisted of a mixture of dairy cream (35% fat) and egg yolk for a total energy content of 766 kcal. The total amount of fat was based on the subject's body surface area (78.3 g fat 55.6 % saturated fatty acids, 29.6 % monounsaturated fatty acids, 4.2 % polyunsaturated fatty acids, 0.5 g cholesterol per m⁻²). The fat load was consumed during a period of 5 min; subjects kept fasting on the test morning and strenuous activity was forbidden, since exercise can reduce postprandial lipemia. Blood samples were drawn every 2 hours for 10 hours and the following parameters were measured in fasting conditions and postprandially at each time of the test:

Oral fat load test: TRLP subfractionation

TRLP were isolated through preparative ultracentrifugation and their total Tg and Chol content were subsequently measured. One aliquot of plasma was brought to densities of 1006 g/L by adding a KBr solution (d=1330 g/L) and centrifuged at 105,000 g for 21h at 10°C in a Beckman L8-70M ultracentrifuge. Tg and total Chol concentration was then determined in lipoprotein fraction

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enzymatically. The first higher amount of blood (10 ml) was drawn for subfractionating TRLPs by ultracentrifugation on a discontinuous density gradient. Separated plasma was brought to a density of 1.10 g/mL by adding solid KBr. The density gradient was prepared by adding to 4 mL of this plasma 3 mL of a 1.065 g/mL solution containing 0.05% KBr/NaCl plus EDTA (pH 7.4); 3 mL of a similar solution at 1.020 g/mL; 3 mL of physiological saline at 1.006 g/mL.

The sample was ultracentrifuged in a Beckman L8-70M centrifuge at 20°C in stages allowing the separation of two VLDL fractions with decreasing Sf values: VLDL 1: Sf>100; VLDL 2: Sf =20-100. The automated methods mentioned above were used to determine Chol and Tg on the two fractions. ApoB48 and ApoB100 content of TRLP subfractions were quantified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Apo B48

Was measured with a sandwich-ELISA as the method. During incubation apo B48 in the sample reacts with anti-apo B48 antibody bound to microtitration well. After washing, which removes non-reactive plasma components, a biotinylated detection antibody specific for apo B48 and avidin-horseradish peroxidase conjugate is added to each micro plate well successively and incubated. After a second incubation and a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with the substrate solution. The reaction is stopped by adding acid to give a colorimetric endpoint, then read spectrophotometrically (Elabscience, China).

Oral fat load test: HDL-C determination

Total HDL-Chol(HDL-C) was determined by enzymatic colorimetric assay after precipitation of LDL and VLDL fractions using heparin-MnCl2 solution and centrifugation at 4°C and it had an intra-assay variation coefficient of 2.5 % and an inter-assay variation coefficient of 4.1%(7).

Oral fat load test: oxLDL measurement

Ox-LDLs were measured with a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies re directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. During incubation oxidized LDL in the sample reacts with anti-oxidized LDL antibodies bound to microtitration well. After washing, which removes non-reactive plasma components, a peroxidase conjugated anti-human apolipoprotein B

antibody recognizes the oxidized LDL bound to the solid phase. Alter a second incubation and a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3,3', 5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint, then read spectrophotometrically (Mercodia, Uppsala, Sweden).

Oral fat load test: Nuclear Factor(NF)-KB activation in mononuclear cells (MNCs)

Blood samples were collected in tubes containing Na-EDTA as an anticoagulant; 3.5 mL of the anticoagulated blood sample was carefully layered over 3.5 mL of the PMNL isolation medium (Robbins Scientific Corp, Sunnyvale, CA). Samples were centrifuged at 450 x g in a swing outrotor for 30 min at 22 °C. At the end of centrifugation, 2 bands separate out at the top of the red blood cell pellet. The top band consists of monocuclear leukocytes(MNCs), whereas the bottom band consists of polymorphumuclear leukocytes(PMNs). The MNC and PMN bands were harvested with a Pasteur pipette, repeatedly washed with Hank's balanced salt solution, and reconstituted to a concentration of 4×10^5 cells/mL in Hank's balanced salt solution. This method yields> 95% pure PMN and MNC suspensions(61).

NF-kBp50/p65 transcription factor assay kit was purchased from Cayman Chemical (Ann Arbor MI, USA). The method detects specific transcription factor DNA binding activity in nuclear extracts and cell lysates. A specific double stranded DNA sequence containing the NF-kB response element is immobilized onto the bottom of wells of a 96 well-plate. NF-kB in nuclear or cytoplasmatic extract binds specifically to the NF-kB response element. NF-kBp50/65 is detected by addition of specific primary antibody directed against NF-kBp50/65. A secondary antibody conjugated to Horseradish peroxidase is added to provide a sensitive colorimetric readout at 450 nm.

Human calprotectin. Human Calprotectin was measured by sandwich ELISA (HyCult Biotechnology sold in Italy by Pantec, Turin, Italy) in plasma samples. Samples and standards are incubated in micro titer wells coated with antibodies recognizing human calprotectin. After this incubation, unbound material in the sample is removed by washing. Biotinylated second antibody (tracer) binds to captured human calprotectin. Excess tracer is removed by washing. Strepatvidin–peroxidase conjugate binds to the biotinylated tracer antibody. Excess of Strepatvidin–peroxidase conjugate is removed by washing

and substrate, tetramethylbenzidine, is added to the wells. Absorbance is read at 450 nm. The calprotectin concentration of samples with unknown concentration can be determined from the standard curve.

Oral fat load test: markers of hepatocyte apoptosis. The M30-Apoptosense ELISA kit, a one step in vitro immunoassay for the quantitative determination of the apoptosis-associated CK18Asp396 neoepitope in serum (PEVIVA AB, Bromma, Sweden), has a sensitivity of 25 U/L in a 25- \Box L sample size and a range of 75 to 1000 U/L. The intra- and inter-assay CVs are less than 8%.

Liver histology in biopsy-proven NAFLD patients. Each pathological feature was read and scored by a single pathologist (RP) blinded to the patient clinical-biochemical characteristics and scored according to the NASH Clinical Research Network criteria; NASH was defined according to Brunt criteria, in line with current guidelines⁶². Steatosis was mild in 34% of NAFLD patients, moderate in 37% NAFLD patients, and severe in 29% patients. Lobular inflammation was absent in 30% patients, grade 1 in 21% patients, grade 2 in 24% patients, and grade 3 in 25% patients. Hepatocyte ballooning was absent in 40% patients, grade 1 in 35% patients and grade 2 in 25% patients. . Fibrosis was stage 0 in 54% patients, 1 in 15% patients, 2 in 18%, and stage 3 in 23% subjects; cirrhotic changes were absent. *Online Appendix Table 1.* Baseline (panel A) and change from baseline (panel B)values in main clinical and biochemical parameters of the follow-up cohort(n=314), grouped according to AGTR1 A/C genotype. In panel B, only parameters that significantly differed across genotypes o changed from baseline during follow-up are shown as Δ (difference between initial and final values)

A)Baseline parameters								
Parameter	AGTR1 AA	AGTR1 AC	AGTR1 CC	Р				
	(n=137)	(n=135)	(n=42)					
Age(yr)	50±1	49±1	49±1	0.784				
Sex(%M)	61	62	60	0.839				
BMI(kg/m ²)	25.8±0.3	25.7±0.3	25.9±0.4	0.721				
Waist (cm)	90±1	89±1	90±1	0.889				
Sys BP(mmHg)	123±2	123±2	124±2	0.813				
Dia BP(mmHg)	81±1	82±2	80±2	0.874				
Family history of T2DM (%)	14	12	8	0.395				
PNPLA3 (%)								
C/C	41	45	41	0.831				
C/G	31	33	42	0.793				
G/G	28	22	17	0.392				
TM6SF2 C/T n(%)								
CC	84	85	84	0.795				
СТ	14	13	13	0.885				
ТТ	2	2	3	0.864				
АроЕ (%)								
2-3	22	20	19	0.731				
3-3	60	60	55	0.713				
3-4	18	20	21	0.791				
Smoking status:								
never(%)	63	62	61	0.612				
former(%)	15	20	22	0.776				
current(%)	22	18	17	0.801				
Education(%):								
Primary school	66	67	65	0.798				
Secondary school	20	17	21	0.801				
University	14	16	14	0.913				

METS(h/week)	22±1	22±1	21±1	0.823
E-selectin (ng/mL)	22.3±3.4	22.5±2.2	24.2±2.9	0.812
ICAM-1 (ng/mL)	201.4±9.6	195.9±9.17	198.6±9.1	0.458
VCAM-1 (ng/mL)	428.7±11.4	435.2±13.2	439.8±13.6	0.639
CRP(mg/L)	1.7±0.1	1.6±0.2	1.6±0.2	0.812
Resistin(ng/mL)	3.4±0.2	3.3±0.3	3.5±0.3	0.839
Adiponectin (ng/mL)	7601±209	7812±300	7791±378	0.693
Nitrotyrosine(nmol/mL)	6.7±1.5	6.3±1.2	6.2±1.5	0.906
TAS(mmol/L)	0.89±0.03	0.90±0.04	0.92±0.02	0.839
Glucose(mg/dL)	93±3	92±3	93±3	0.793
HOMA-IR	1.5±0.2	1.6±0.3	1.5±0.4	0.802
Tg(mg/dL)	113±8	114±9	113±10	0.839
Total C(mg/dL)	187±5	184±7	186±9	0.748
HDL-C(mg/dL)	61±2	60±2	60±4	0.872
AST(IU/L)	16±1	16±2	17±2	0.862
ALT(IU/L)	18±1	17±2	19±2	0.827
	B)Change fro	om baseline		
Δ Sys BP(mmHg)	+6±1	+12±2	+20±2	0.007
Δ Dia BP(mmHg)	+3±1	+6±1	+10±1	0.009
Δ CRP(mg/L)	0.6±0.2*	1.2±0.2*	2.3±0.1**	0.010
Δ HOMA-IR	0.5±0.2*	1.1±0.2*	1.9±0.1*	0.018
ΔE-selectin (ng/mL)	4.1±1.1**	10.1±1.9**	18.94±1.9**	0.007
Δ ICAM-1 (ng/mL)	9.3±2.1**	37.4±5.2**	82.3±6.9**	0.001
Δ vCAM-1 (ng/mL)	79.3±6.1**	112.4±5.2**	192.3±8.1**	0.001
Δ Resistin(ng/mL)	0.6±0.1	1.0±0.2	1.9±0.3	0.038
Δ Adiponectin (ng/mL)	-621±41*	-813±62*	-1939±181*	0.009
Δ Nitrotyrosine(nmol/mL)	5.5±1.1	9.2±1.1	16.4±1.2	0.026
Δ TAS(mmol/L)	-0.26±0.01*	-0.38±0.03*	-0.58±0.01*	0.036
Δ AST(IU/L)	3±2**	14±2**	29±1**	0.008
Δ ALT(IU/L)	4±3**	12±2**	29±1**	0.0002
Incident NAFLD n(%)	22(16) **	37(27) *	22(52) ‡	0.020
Incident hypertension n(%)	40(29%)0	58(43%)	26(62%)	0.020
Incident T2DM n(%)	7(5) **	8(6) *	3(7) ‡	0.371
Incident Obesity n(%)	10(7) **	14(10) **	4(10) **	0.719

The P values value refer to comparison between groups at baseline, at the end of follow-up and to comparison in changes during the follow-up, respectively. Data are expressed as mean±SEM. Subjects with different AGTR1 A/C genotypes were compared for baseline values and for changes during follow-up. Normality was evaluated by Shapiro-Wilk test. Fisher or chi-square test were used to compare categorical variables, as appropriate.

Differences across groups were analyzed by ANOVA and then by Bonferroni correction, when variables were normally distributed; otherwise the Kruskal-Wallis test, followed by the post hoc Dunn test, was used to compare nonparametric variables. To adjust for multiple comparison testing, the Benjamini-Hochberg False Discovery Rate correction was applied to raw p-values; significance was set at an adjusted p-value threshold of 0.05

Abbreviations: BP: blood pressure; C: cholesterol; CRP: C-reactive protein; Dia: diastolic; HOMA-IR: homeostasis model assessment of insulin resistance; ICAM: intercellular adhesion molecule; IGR: impaired glucose regulation; METS: Metabolic equivalent of activity (h/week); PNPLA3: patatin-like phospholipase domain-containing 3; Sys: systolic; T2DM: type 2 diabetes mellitus; Tg: triglycerides;

* p<0.05 vs. all other AGTR1 genotypes
** p<0.01 vs. all other AGTR1 genotypes
§ p<0.001 vs. all other AGTR1 genotypes
† p<0.05 vs. AGTR1 AC genotype
‡ p<0.01 vs. AGTR1 AC genotype
p<0.001 vs. AGTR1 AC genotype
¶ p<0.05 vs. AGTR1 AA genotype
¶ ¶ p<0.001 vs. AGTR1 AA genotype

REFERENCES

[1] Mari A, Pacini G, Murphy E. A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. Diabetes Care 2001; 24: 539-548.

[2] Abdul-Ghani MA, Matsuda M, Balas B. Muscle and liver insulin resistance indexes derived from the oral glucose tolerance test. Diabetes Care. 2007; 30:89-94.

[3] Thomaseth K, Kautzky-Willer A, Ludvik B. Integrated mathematical model to assess beta-cell activity during the oral glucose test. Am J Physiol. 1996; 270: E522-31

[4] Tura A, Ludvik B, Nolan JJ. Insulin and C-peptide secretion and kinetics in humans: direct and model-based measurements during OGTT. Am J Physiol Endocrinol Metab. 2001;28: E966-74.

[5] Tura A, Kautzky-Willer A, Pacini G. Insulinogenic indices from insulin and C-peptide:

comparison of beta-cell function from OGTT and IVGTT. Diabetes Res Clin Pract 2006; 72: 298-301.

[6]Abdul-Ghani MA, Williams K, DeFronzo RA. What is the best predictor of future type 2 diabetes? Diabetes Care 2007; 30:1544-8.

[7] Warnick GR, Albers JJ. A comprehensive evolution of the heparin manganese precipitation procedure for estimating high density lipoprotein cholesterol, J. Lipid Res. 1978; 29: 65-76.

[8] Gidez, L.I., Miller, G.J., Burnstein, M., Slagle, S., and Eder, H.A. Separation and quantitation of subclasses of human plasma HDL by a single precipitation procedure, J. Lipid Res. 1982; 23: 1206-16.

[9]Stocks SG, Miller NE. Capillary electrophoresis to monitor the oxidative modification of LDL. J Lipid Res 1998; 39: 1305-09.

[10] Kleiner DE, Brunt EM, Van Natta M. Design and Validation of a Histological Scoring System for Nonalcoholic Fatty Liver Disease.Hepatology 2005; 41:1313-21

[11] Brunt EM. Nonalcoholic steatohepatitis: definition and pathology. Semin Liver Dis 2001;21:3-16.

REFERENCES

[1]Kabbany MN, Conjeevaram Selvakumar PK, Watt K, et al. Prevalence of Nonalcoholic Steatohepatitis-Associated Cirrhosis in the United States: An Analysis of National Health and Nutrition Examination Survey Data. Am J Gastroenterol. 2017 ;112:581-587.

[2]Musso G, Cassader M, Gambino R, et al. Meta-analysis: Natural history of NAFLD and diagnostic accuracy of non-invasive tests for liver disease severity. Ann Med. 2011;43:617-49

[3] Mehta N, Singh T, Lopez R,, et al. The Heart Age Is Increased in Patients With Nonalcoholic Fatty Liver Disease and Correlates With Fibrosis and Hepatocyte Ballooning. Am J Gastroenterol. 2016;111:1853-1854.

[4]Aneni EC, Oni ET, Martin SS, et al. Blood pressure is associated with the presence and severity of nonalcoholic fatty liver disease across the spectrum of cardiometabolic risk. J Hypertens. 2015;33:1207-14.

[5]Vlachopoulos C, Manesis E, Baou K, et al. Increased arterial stiffness and impaired endothelial function in nonalcoholic Fatty liver disease: a pilot study. Am J Hypertens. 2010;23:1183-9.

[6]Musso G, Cassader M, Gambino R. Non-alcoholic steatohepatitis: emerging molecular targets and therapeutic strategies. Nat Rev Drug Discov. 2016;15:249-74

[7]Schwab U, Lauritzen L, Tholstrup T. Effect of the amount and type of dietary fat on cardiometabolic risk factors and risk of developing type 2 diabetes, cardiovascular diseases, and cancer: a systematic review. Food Nutr Res. 2014;58.

[8]Bo S, Musso G, Beccuti G, et al. Consuming more of daily caloric intake at dinner predisposes to obesity. A 6-year population-based prospective cohort study. PLoS One. 2014;9:e108467.

[9]Oddy WH, Herbison CE Jacoby P, et al. The Western dietary pattern is prospectively associated with nonalcoholic fatty liver disease in adolescence. Am J Gastroenterol. 2013;108:778-85.

 [10] Krawczyk M, Rau M, Schattenberg JM, et al.Combined effects of the PNPLA3 rs738409,
 TM6SF2 rs58542926, and MBOAT7 rs641738 variants on NAFLD severity: a multicenter biopsybased study. J Lipid Res. 2017;58:247-255. [11] Umano GR, Caprio S, Di Sessa A, et al. The rs626283 Variant in the MBOAT7 Gene is Associated with Insulin Resistance and Fatty Liver in Caucasian Obese Youth.Am J Gastroenterol. 2018;113:376-383.

[12]Souza-Mello V. Hepatic structural enhancement and insulin resistance amelioration due to AT1 receptor blockade. World J Hepatol. 2017;9:74-79

[13]Namisaki T, Moriya K, Kitade M, et al.Effect of combined farnesoid X receptor agonist and angiotensin II type 1 receptor blocker on hepatic fibrosis. Hepatol Commun. 2017;1:928-945..

[14]Goh GB, Pagadala MR, Dasarathy J, et al. Renin-angiotensin system and fibrosis in non-alcoholic fatty liver disease. Liver Int. 2015;35:979-85.

[15]Pelusi S, Petta S, Rosso C, et al. Renin-Angiotensin System Inhibitors, Type 2 Diabetes andFibrosis Progression: An Observational Study in Patients with Nonalcoholic Fatty Liver Disease.PLoS One. 2016 Sep 20;11: e0163069.

[16]Sethupathy P, Borel C, Gagnebin M, et al. Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3' untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. Am J Hum Genet. 2007;81:405-13

[17] Yang Y, Tian T, Lu J, et al. A1166C polymorphism of the angiotensin II type 1 receptor gene contributes to hypertension susceptibility: evidence from a meta-analysis. Acta Cardiol. 2017;72:205-215.

[]18Feng X, Zheng BS, Shi JJ, et al. A systematic review and meta-analysis of the association between angiotensin II type 1 receptor A1166C gene polymorphism and myocardial infarction susceptibility. J Renin Angiotensin Aldosterone Syst. 2014;15:307-15.

[19]Palatini P, Ceolotto G, Dorigatti F, et al. Angiotensin II type 1 receptor gene polymorphism predicts development of hypertension and metabolic syndrome. Am J Hypertens. 2009;22:208-14.
[20]Milionis HJ, Kostapanos MS, Vakalis K, et al. Impact of renin-angiotensin-aldosterone system genes on the treatment response of patients with hypertension and metabolic syndrome. J Renin Angiotensin Aldosterone Syst. 2007; 8:181-9.

[21] Sun Y, Liao Y, Yuan Y, et al. Influence of autoantibodies against AT1 receptor and AGTR1 polymorphisms on candesartan-based antihypertensive regimen: results from the study of optimal treatment in hypertensive patients with anti-AT1-receptor autoantibodies trial. J Am Soc Hypertens. 2014;8:21-7.

[22] https://www.genecards.org) accessed on June 20th, 2017

[23] https://www.snpedia.com/index.php/ accessed on June 20th, 2017

[24] Musso G, Cassader M, Bo S, et al. Sterol regulatory element-binding factor 2 (SREBF-2) predicts 7-year NAFLD incidence and severity of liver disease and lipoprotein and glucose dysmetabolism. Diabetes. 2013;62:1109-20

[25]Miyake T, Kumagi T, Hirooka M. Metabolic markers and ALT cutoff level for diagnosing nonalcoholic fatty liver disease: a community-based cross-sectional study. J Gastroenterol. 2012; 47: 696-703.

[26]Webb M, Yeshua H, Zelber-Sagi S. Diagnostic value of a computerized hepatorenal index for sonographic quantification of liver steatosis. *AJR Am J Roentgenol* 2009;192:909–914.

[27] James PA, Oparil S, Carter BL, et al. 2014 evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8) JAMA 2014;311:507-20.

[28]Musso G, Gambino R, De Michieli F, et al. Nitrosative stress predicts the presence and severity of nonalcoholic fatty liver at different stages of the development of insulin resistance and metabolic syndrome: possible role of vitamin A intake. Am J Clin Nutr. 2007;86:661-71.

[29]Alberti KG, Eckel RH, Grundy SM, et al; Harmonizing the metabolic syndrome: a joint interim statement of the IDF Task Force on Epidemiology and Prevention, NHLBI, AHA, WHF, IAS, and IASO. Circulation 2009 ;120: 1640-5

[30]Taylor HL, Jacobs DR Jr, Schucker B. Questionnaire for the assessment of leisure time physical activities. J Chronic Dis 1978; 31: 741–755.

[31]Akpinar TS, Ozkok A, Kose M, et al. Endothelial constitutive nitric oxide synthase, angiotensin converting enzyme, angiotensin II type 1 receptor gene polymorphisms and endothelial functions in healthy individuals. Eur Rev Med Pharmacol Sci. 2014;18: 39-45.

[32]Ridker PM, Hennekens CH, Roitman-Johnson B. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998; 351: 88-92.

[33] Lefere S, Van de Velde F, Devisscher L, et al. Serum vascular cell adhesion molecule-1 predicts significant liver fibrosis in non-alcoholic fatty liver disease. Int J Obes (Lond). 2017;41:1207-1213.

[34] Pahlavani M, Kalupahana NS, Ramalingam L, et al. Regulation and Functions of the Renin-Angiotensin System in White and Brown Adipose Tissue. Compr Physiol. 2017;7:1137-1150

[35]Zimnol A, Amann K, Mandel P, et al. Angiotensin II type 1a receptor-deficient mice develop angiotensin II-induced oxidative stress and DNA damage without blood pressure increase. Am J Physiol Renal Physiol. 2017 Sep 6:ajprenal.00183.2017. doi: 10.1152/ajprenal.00183.2017. [Epub ahead of print]

[36]Abdelmegeed MA, Song BJ. Functional roles of protein nitration in acute and chronic liver diseases. Oxid Med Cell Longev. 2014;2014:149627.

[37]Musso G, Cassader M, De Michieli F. Nonalcoholic steatohepatitis versus steatosis: adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. Hepatology. 2012;56: 933-42

[38]Klop B, van den Berg TM, Rietveld AP, et al AT1 Receptor Gene Polymorphisms in relation to Postprandial Lipemia. Int J Vasc Med. 2012; 2012: 271030.

[39]Cobelli C, Toffolo GM, Dalla Man C. Assessment of beta-cell function in humans, simultaneously with insulin sensitivity and hepatic extraction, from intravenous and oral glucose tests. Am J Physiol Endocrinol Metab. 2007; 293: E1-E15.

[40]Higgins V, Adeli K. Postprandial Dyslipidemia: Pathophysiology and Cardiovascular DiseaseRisk Assessment. EJIFCC. 2017;28:168-184

[41]Yasu T, Mutoh A, Wada H, et al. Renin-Angiotensin System Inhibitors Can Prevent Intravenous Lipid Infusion-Induced Myocardial Microvascular Dysfunction and Leukocyte Activation. Circ J.
2017 Sep 26. doi: 10.1253/circj.CJ-17-0809. [Epub ahead of print]

[42] Borghi C, Urso R, Cicero AF.Renin-angiotensin system at the crossroad of hypertension and hypercholesterolemia. Nutr Metab Cardiovasc Dis. 2017 27:115-120.

[43]Wu Y Ma KL, Zhang Y, et al Lipid disorder and intrahepatic renin-angiotensin system activation synergistically contribute to NAFLD.Liver Int. 2016;36:1525-34.

[44]Nakamura K, Matsui T, Adachi H, et al. Involvement of angiotensin II in intestinal cholesterol absorption. Pharmacol Res. 2010;61:460-5

[45] Inoue T, Taguchi I, Abe S, et al. Inhibition of intestinal cholesterol absorption might explain cholesterol-lowering effect of telmisartan. J Clin Pharm Ther. 2011;36:103-10

[46]Michel MC, Brunner HR, Foster C, et al. Angiotensin II type 1 receptor antagonists in animal models of vascular, cardiac, metabolic and renal disease. Pharmacol Ther. 2016;164:1-81.

[47]Muñoz A, Costa M. Nutritionally mediated oxidative stress and inflammation.

Oxid Med Cell Longev. 2013;2013:610950

[48]Tan DY, Shi HY, Li CP, et al. Effect of nuclear factor-κB and angiotensin II receptor type 1 on the pathogenesis of rat non-alcoholic fatty liver disease. World J Gastroenterol. 2015;21: 5877-83.
[49] Kruzliak , Novák J, Novák M, et al. Role of calprotectin in cardiometabolic diseases. Cytokine Growth Factor Rev. 2014;25:67-75.

[50]Wu Y, Li Y, Zhang C, et al. S100a8/a9 released by CD11b+Gr1+ neutrophils activates cardiac fibroblasts to initiate angiotensin II-Induced cardiac inflammation and injury. Hypertension.
2014;63:1241-50.

[51]Mukai K, Miyagi T, Nishio K, et al. S100A8 Production in CXCR2-Expressing CD11b+Gr-1high Cells Aggravates Hepatitis in Mice Fed a High-Fat and High-Cholesterol Diet. J Immunol. 2016;196:395-406. [52]Dordevic AL, Pendergast FJ, Morgan H. Postprandial Responses to Lipid and CarbohydrateIngestion in Repeated Subcutaneous Adipose Tissue Biopsies in Healthy Adults. Nutrients.2015;7:5347-61.

[53]Arsov T, Carter CZ, Nolan CJ. Adaptive failure to high-fat diet characterizes steatohepatitis in Alms1 mutant mice. Biochem Biophys Res Comm 2006; 342: 1152-1159
[54]Musso G, Gambino R, Cassader M, et al. Specialized Proresolving Mediators: Enhancing Nonalcoholic Steatohepatitis and Fibrosis Resolution. Trends Pharmacol Sci. 2018;39:387-401

[55]Foster T, Budoff MJ, Saab S, et al. Atorvastatin and antioxidants for the treatment of nonalcoholic fatty liver disease: the St Francis Heart Study randomized clinical trial. Am J Gastroenterol. 2011;106:71-7.

[56] Musso G, Cassader M, Paschetta E, et al. Thiazolidinediones and Advanced Liver Fibrosis in Nonalcoholic Steatohepatitis: A Meta-analysis. JAMA Intern Med. 2017; 177:633-640

[57]Ruiz-Hurtado G, Banegas JR, Sarafidis PA, et al. Has the SPRINT trial introduced a new bloodpressure goal in hypertension? Nat Rev Cardiol. 2017;14:560-566.

[58] Musso G, De Michieli F, Bongiovanni D, et al. New Pharmacologic Agents That TargetInflammation and Fibrosis in Nonalcoholic Steatohepatitis-Related Kidney Disease.Clin Gastroenterol Hepatol. 2017;15:972-985

ACKNOWLEDGEMENTS

Sources of support: Grant ex-60%, 2013, Dept of Medical Sciences, University of Turin Conflicts of interest: no author has any present or past conflict of interest or financial interest to disclose

Guarantor of the article: Giovanni Musso **Author's contributions** Giovanni Musso: conceived and designed the study, analyzed the results, drafted the article, gave final approval

Francesca Saba: performed biochemical analyses, critically analyzed the results, contributed to the draft of the article, gave final approval

Maurizio Cassader: acquired data, critically analyzed the results, contributed to the draft of the article, gave final approval

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⁵⁹]Sacerdote C, Fiorini L, Dalmasso M. Alimentazione e rischi di cancro. Indagine su un

campione di 10054 volontari residenti nell'area torinese. Torino: AGAT 2000.

[60]Carnovale E, Marletta P. Food composition table. Istituto Nazionale della

Nutrizione. Milano: EDRA 1997.

[⁶¹]Ghanim H, Abuaysheh S, Sia CL. Increase in plasma endotoxin concentrations and the expression of Toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells following a high-fat high-carbohydrate meal: implications for insulin resistance. Diabetes Care 2009;32:2281–2287
 ⁶² Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM,

Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from

the American Association for the Study of Liver Diseases. Hepatology. 2018;67:328-357