# METABOLISM, RENAL INSUFFICIENCY AND LIFE EXPECTANCY

Studies on obesity, chronic kidney diseases and aging

**Belinda Gilda Spoto**

**Cover:** “Né più mi occorrono, le coincidenze, le prenotazioni, le trappole, gli scorni di chi crede che la realtà sia quella che si vede“ (*Eugenio Montale, Satura 1962-70*)

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# METABOLISM, RENAL INSUFFICIENCY AND LIFE EXPECTANCY

Studies on obesity, chronic kidney diseases and aging

**Metabolisme, nierinsufficiëntie en levensverwachting**

**Studies over obesitas, chronische nierziekten en veroudering**

***Proefschrift***

ter verkrijging van de graad van doctor aan de

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*A Piero, il mio "approdo" sempre*

*A Michela per avermi insegnato a scalare le montagne*

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# CHAPTER 1

General introduction and outline of the thesis

**GENERAL INTRODUCTION**

Currently, chronic diseases are well recognized as major contributors to global mortality (1) and, by 2030, it is expected that these diseases will account for more than three-quarters of deaths worldwide. Within chronic diseases, cardiovascular disease (CVD) emerged as the leading cause of global mortality (2). In two decades, the total number of cardiovascular (CV) deaths raised from 14.4 million to 17.5 million and achieved nearly 20 million in 2015, accounting for 31% of all deaths worldwide (2). CVD is thought to be a problem of wealthy nations, whereas infectious diseases are considered the main cause of mortality in developing countries. However, a large body of epidemiological evidence showed that in low and middle income nations, CVD is responsible for more deaths than infectious diseases, poor maternal/perinatal conditions and nutritional disorders combined (3). Thus, CVD can be considered as the largest simple contributor to global mortality and, according to the World Health Organization (WHO), CVD will continue to dominate mortality trends in the close future (4). The worsening of CV health around the world reflects significant global changes in behavior and lifestyle. The "westernization" of dietary habits and decreased physical activity are now practices that also threaten developing countries. In addition, the decline in infectious diseases and improved childhood nutrition have contributed to the aging of populations resulting in an increasing number of individuals who survive to the age at which risk factors they accrued throughout childhood and early adulthood, manifest as overt disease. This has resulted in an epidemic of CVD in the developing countries comparable to the one that took place in the developed world in previous decades: CVD has global dimensions.

Over the past years, a considerable amount has been learned about the determinants of CVD and a series of both modifiable and non-modifiable risk factors have been identified. Several risk factors [i.e. age, male gender, high levels of low density lipoprotein (LDL) cholesterol, smoking, diabetes, hypertension and family history of CVD] emerged from the Framingham Study (5) and are now well-recognized risk factors for CVD. However, these "traditional" risk factors only identify 70% of

individuals at risk for CV events pointing at new factors contributing to CVD development (6). Insulin resistance, inflammation and oxidative stress are emerging risk factors of paramount importance in CV risk, heavily affecting CV morbidity and mortality (7, 8, 9). They are closely related to pathophysiological processes, each of them being cause and consequence of the others in a self-perpetuating vicious cycle. The strict interconnection among them makes difficult to disentangle the effect of the single risk factor on CV system components. As experimental and epidemiologic research indicates, a close association between reactive oxygen species (ROS) and chronic inflammation exists (9). ROS can trigger the production of pro-inflammatory cytokines (TNFα, IL-1, IL-6), chemokines (IL-8) and pro-inflammatory transcription factors (NF-κB) (10) but, on the other hand, inflammation promotes oxidative stress (11). Oxidative stress can also lead to insulin resistance (12, 13, 14) but, at the same time, metabolic derangements induce oxidative stress and compromise inflammatory response (15) that, in turn, can causes alterations in insulin signaling pathway (13, 16). Insulin resistance, inflammation and oxidative stress are alterations that characterize a variety of chronic diseases. Impaired insulin sensitivity and subclinical low-grade inflammation are pervasive conditions in obesity (17, 18) and chronic kidney disease (19, 20) while oxidative stress is the main responsible for aging (21).

**OUTLINE OF THE THESIS**

The aim of this thesis is to investigate whether insulin resistance, inflammation and oxidative stress increase CV risk and affect survival in high-risk populations. To address this question, epidemiologic and genetic studies were carried out in obese individuals, elderly and patients with chronic kidney disease of various severity, who represent three populations with high CV risk.

Schematically, the thesis is divided in two parts: *Part I*, which reports results from two cross-sectional studies, and *Part II*, which shows results from four prospective studies. Coming up, the findings are placed into perspective in the general discussion where

suggestions for future research are also addressed and, finally, a summary gives an overview of the thesis.

In short:

In **Chapter 2**, the expression profiles of pro-inflammatory and anti-inflammatory genes in abdominal subcutaneous and visceral adipose tissue in severely obese individuals are investigated to assess the specific contribution to inflammation of the two fat depots. It is recognized that important differences exist in the gene expression profile of subcutaneous and visceral adipose tissue but, with respect to inflammatory genes, results are controversial. In this respect, the basic hypothesis is that topography of adipose tissue accumulation is relevant for the risk of developing inflammation and, in turn, of enhancing the risk of CV complications.

In **Chapter 3**, the hypothesis that genetic markers of insulin resistance modify the link between a pro-fibrotic cytokine at myocardial level, i.e. TIMP-1, and left ventricular (LV) mass and function in a group of dialysis patients is investigated. The background is based on two observations: 1) insulin resistance promotes myocardial fibrosis; 2) the genetic markers considered in this study were previously associated with LV hypertrophy in the same population of patients.

In **Chapter 4**, the nature (causal vs non-causal) of the association between IL-6 and fatal and non-fatal CV event in a population of patients with CKD of various severity is determined by using the approach of Mendelian randomization to infer causality in an observational setting.

**Chapter 5** shows the results of a genetic association study testing whether the variability of the FTO gene contributes to explain mortality in 3 cohorts of patients with CKD of various severity. The issue is of relevance because diabetes and hypertension, two risk factors which have been associated to the FTO gene, rank as major risk factors for CKD and survival in this population. Results are presented as independent data referring to each cohort and as pooled data analyzed by a meta-analytic approach.

**Chapter 6** reports the results of a study focused on investigating the mutual relationship between resistin and the two major adipokines (i.e. adiponectin and leptin) and addressing the potential interaction between resistin and adiponectin on all-cause and CV mortality in a cohort of patients with kidney failure.

**Chapter 7** shows the results of an observational longitudinal study performed in a population-based cohort of elderly individuals (>65 years) from the Invecchiare in Chianti study and aimed at: 1) investigating the relationship between gamma-glutamyltransferase (GGT), a multifaceted biomarker impinging upon oxidative stress, and all-cause and CV mortality; 2) assessing whether oxidized low-density lipoproteins (oxLDL), which co-localize with GGT in atherosclerotic plaques, modify the relationship between GGT and mortality.

**Chapter 8** presents a general discussion and alludes to future perspectives.

**Chapter 9** reports a compendium of the thesis.

**Chapter 10** is the summary of the thesis in Dutch.

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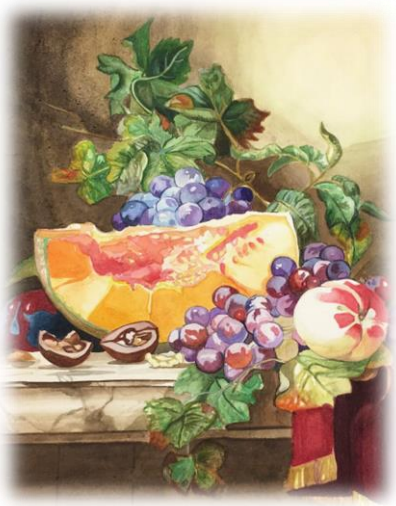
# PART I

## Insulin resistance, inflammation and oxidative stress in obesity and kidney disease: a lesson from cross-sectional studies



## CHAPTER 2

# Pro- and anti-inflammatory cytokine gene expression in subcutaneous and visceral fat in severe obesity

Spoto B, Di Betta E, Mattace-Raso F, Sijbrands E, Vilardi A, Parlongo RM, Pizzini P, Pisano A, Vermi W, Testa A, Cutrupi S, D'Arrigo G, Lonardi S, Tripepi G, Cancarini G, Zoccali C.

*Nutr Metab Cardiovasc Dis. 2014;24:1137-43*

**ABSTRACT**

**Background and Aims:** Pro-inflammatory molecules produced by adipose tissue have been implicated in the risk of cardiovascular (CV) disease in obesity. We investigated the expression profile of 19 pro-inflammatory and 7 anti-inflammatory genes in subcutaneous adipose tissue (SAT) and in visceral adipose tissue (VAT) in 44 severely obese individuals who underwent bariatric surgery.

**Methods and Results:** SAT and VAT expressed an identical series of pro-inflammatory genes. Among these genes, twelve were significantly more expressed in SAT than in VAT while just one (IL18) was more expressed in VAT. The remaining genes were equally expressed. Among pro-inflammatory cytokines, both IL6 and IL8 were about 20 times more intensively expressed in SAT than in VAT. The expression of nine genes was highly associated in SAT and VAT. Only for 3 pro-inflammatory cytokines (IL8, IL18, SAA1) in SAT the gene expression in adipose tissue associated with the circulating levels of the corresponding gene products while no such an association was found as for VAT.

**Conclusions:** The expression of critical pro-inflammatory genes is substantially higher in SAT than in VAT in individuals with morbid obesity. The variability in circulating levels of pro-inflammatory cytokines is, in small part and just for three pro-inflammatory cytokines, explained by underlying gene expression in SAT but not in VAT.

These results point to a compartment-specific adipose tissue contribution to inflammation in obesity and indicate that abdominal SAT contributes more than VAT to the pro-inflammatory milieu associated with severe obesity.

**INTRODUCTION**

Adipose tissue is distributed throughout the body in discrete fat compartments which broadly cluster into two regions, a central and a peripheral one (1). The central region includes subcutaneous adipose tissue (SAT) of the thorax and the abdomen as well as intra-thoracic and intra-abdominal visceral adipose tissue (VAT), while peripheral fat consists of subcutaneous fat depots in the arms and the legs. The topography of adipose tissue accumulation is considered relevant for the risk of developing the metabolic and hemodynamic sequels of insulin resistance, including type 2 diabetes, dyslipidaemia and hypertension (2) but the issue remains controversial. Waist to hip circumference ratio, an established metric of abdominal obesity, consistently associates with hyperinsulinemia, glucose intolerance, type 2 diabetes, dyslipidaemia, hyperuricemia and cardiovascular disease (3). However, a large waist to hip ratio may encompass both increased SAT and VAT depots and therefore this metric does not allow a distinction of the underlying links of visceral and subcutaneous fat with hyperinsulinemia and attendant metabolic alterations. The issue is of relevance because VAT is generally held as the main determinant of metabolic risk (4) while SAT is considered either neutral or protective as for the same risk (5). In VAT, free fatty acids (FFA) generated by enhanced lipolysis directly augment lipid synthesis and gluconeogenesis in the liver thereby triggering insulin resistance, hypertension and atherosclerosclerotic complications (4). However, visceral fat is just a minor segment of total fat depots (less than 1/5 of whole body fat tissue) contributing to about 15% of the whole body FFA pool which is made up mainly by non-splanchnic adipose tissue (3, 6).

Adipose tissue is also an abundant source of inflammatory cytokines and an excess of fat mass has been associated with a chronic subclinical inflammatory state (7). It is recognized that important differences exist in the gene expression profile of abdominal SAT and VAT (8-10) and that these two fat depots independently enhance the risk of CV complications (11). However, with respect to inflammatory genes, only few studies explored a large set of pro-inflammatory and anti-inflammatory cytokines

(12, 13) and the results are controversial. Further studies encompassing multiple inflammatory genes in SAT and VAT are of obvious relevance to clarify the relative role of these two adipose tissue compartments in fat-dependent inflammatory mechanisms in human obesity. With this background in mind, we compared the expression profiles of 19 major pro-inflammatory and 7 anti-inflammatory genes in SAT and VAT in 44 severely obese individuals.

## SUBJECTS AND METHODS

The protocol of the study was approved by the local ethical committee and all subjects gave informed, written consent to their participation into the study.

### *Subjects*

The study population was recruited from the Division of General Surgery 1 of Brescia and included 44 incident obese patients who underwent bariatric surgery (biliopancreatic diversion in 11; gastric bypass in 10; mini-gastric bypass in 22; abdominal plastic in 1).

### *Laboratory measurements*

Blood sampling was performed early in the morning after an overnight fast and plasma was stored at - 80°C until batch analyses. Serum glucose, cholesterol, triglycerides, albumin, haemoglobin, urea, uric acid, bilirubin, GOT, GPT, creatinine and C-reactive protein measurements were made using standard methods implemented in a multichannel analyser in the routine clinical laboratory. Insulin (MP Biomedicals, NY, USA) as well as adiponection and leptin (Linco Reasearch, USA) were measured by radioimmunoassay kits. Enzyme-linked immunosorbent assays (ELISA) were applied to measure plasma levels of IL1$\beta$, IL6, TNF$\alpha$, IL18, resistin, PAI, VCAM1 (R&D Systems, Inc., Minneapolis, USA), IL8, SAA1 (Invitrogen, Carlsbad, CA, USA) and visfatin (Adipogen International, Inc., San Diego, USA).

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according the formula HOMA-IR=[fasting insulin concentration ($\mu$U/mL) x fasting glucose concentration (mmol/L)]/22.5.

**Adipose tissue sampling and gene expression analysis**

SAT and VAT from abdominal region was harvested at the beginning of surgical intervention and the adipose samples were collected in RNAlater (Ambion, Life Technologies, USA) and stored at -80°C until processing for RNA extraction. Total RNA was isolated from approximately 80-mg frozen SAT and VAT by means of the RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, USA), according to the manufacturer's instructions. Total RNA was treated with the DNA-free kit (Ambion, Austin, TX, USA) to digest contaminating genomic DNA. The concentration of the RNA samples was determined spectrophotometrically (NanoDrop ND-1000, Thermo Fisher Scientific Inc.). Single-stranded complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Pre-validated TaqMan Gene Expression Assays from Applied Biosystems were used to quantify the expression of pro-inflammatory genes (IL6, IL6R, IL8, CXCR1, CXCR2, TNF$\alpha$, IL1$\beta$, IL1R1, TGF$\beta$, MCP1, IL18, PAI, SAA1, TLR4, ICAM1, VCAM1, Visfatin, Resistin, Leptin) and anti-inflammatory genes (IL2, IL4, IL10, IL13, SOCS3, CD163, Adiponectin). The RT-PCR was performed by a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All genes were run in duplicate and negative controls were introduced in each plate. Target genes were considered unexpressed if the threshold cycle (Ct) value $\geq$ 38. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene expression to correct for variation in RNA amounts and efficiency of reverse transcription. The relative quantification value of the target genes was calculated using the comparative Ct method, expressed as $2^{-[delta][delta]Ct}$ (fold difference), and reported as arbitrary units (AU).

***Gene expression in pooled samples***

To preliminary test the gene expression of the 26 target genes, we performed a pooling analysis using a SAT and VAT pool. Each pool was built using an identical quantity of SAT and VAT mRNA from every patient. Pooled mRNA was reverse transcribed and the resulting cDNA was amplified. The gene expression of the target

genes in the two pools was compared and only those differentially expressed in SAT and VAT were further analysed on individual basis. To identify differentially expressed target genes we adopted a conservative approach consisting of a difference in SAT/VAT gene expression ratio more than 50%.

***Histological analysis* of *adipose tissue samples***

SAT and VAT samples obtained from 14 patients were formalin-fixed and paraffin-embedded. Four micron tissue sections were stained for hematoxylin and eosin and immunostained using a Bond MaxTM autostainer (Menarini Diagnostics, Florence, Italy). Standard immunoperoxidase staining protocols for CD45 - a pan-leukocyte antigen - (Clone RP2/18 and RP2/22, Leica Microsystems, Newcastle upon Tyne, UK) and CD163 – an antigen for a macrophage subpopulation of major relevance for the anti-inflammatory response - (Clone 10D6, Thermo Scientific, Fremont, CA) were followed. In all the adipose tissue samples, cell automatic counting on CD163 stained sections was performed on digitalized slides (Aperio Scanscope, CA, USA) by analyzing the whole section using IHC Nuclear algorithm. Data were expressed as number of cells/$cm^2$.

***Statistical analysis***

Data are expressed as mean ± SD (normally distributed data), median and inter-quartile range (non-normally distributed data) or as percent frequency, as appropriate. Within groups comparisons were made by the Wilcoxon Rank test. The association between two continuous variables considered simultaneously was assessed by Pearson product moment correlation coefficients (r) and P values. Variables having a positively skewed distribution were log transformed (Ln) before the correlation study. The correlation coefficient was calculated with and without the exclusion of the outliers as identified by Mahalanobis distance test (14). The agreement between gene expression in VAT and SAT was investigated by calculating the shared variance ($r^2$) of tested genes in visceral and subcutaneous fat. Because our study focuses on a specific etiological hypothesis and on a strong a priori we did not account for multiple testing (15).

***Study power***

In a previous paper in severe obese women (13), the ratio between SAT and VAT gene expression of a major inflammatory biomarker (IL6) was reported to be 3. With this background in mind, we calculated that by enrolling at least 44 obese individuals (including a 10% attrition rate) our study will achieve 80% power to detect as significant (alpha-error=0.01) a ratio $\geq$3 in IL6 gene expression between SAT and VAT. We assumed that the ratio in the gene expression between SAT and VAT of all inflammatory and anti-inflammatory molecules considered in the study was equal or greater of that calculated for IL6.

**RESULTS**

***Demographic, somatometric and clinical characteristics of the patients***

Demographic, somatometric and clinical characteristics of the patients are reported in **Table 1**.

The mean age of the patients was 41±9 years (11 M and 33 F). Obesity was of grade I (BMI ranging from 30.0 to 34.9 kg/m$^2$) in 4 cases (9%), of grade 2 (BMI ranging from 35.0 to 39.9 kg/m$^2$) in 12 cases (27%) and of grade III in the remaining 28 cases (64%). The median value of glycemia was 99 mg/dL and only 4 were diabetic (3 on oral hypoglycemic drugs and 1 on insulin treatment). Serum cholesterol was on average 199 mg/dL and was above the upper limit of the normal range (200 mg/dL) in 22 cases. Blood pressure (BP) was 127±8/79±8 mmHg. No patient had a BP exceeding 140/90 mmHg and only 3 were on anti-hypertensive treatment (2 on mono-therapy with sartans or β-blockers or angiotensin-converting enzyme inhibitors and the remaining one on triple therapy with a β-blocker, an angiotensin-converting enzyme inhibitor and a diuretic). Six patients were habitual smokers. None of the patients was suffering from cancer, thyroid disease, liver disease or acute infections.

**Table 1.** Main demographic, somatometric and clinical characteristics of the study patients

| | **(n = 44)** |
|---|---|
| Age (years) | 41±9 |
| Male sex n. (%) | 11 (25) |
| BMI (kg/m$^2$) | 43±7 |
| Diabetics n. (%) | 4 (9) |
| Smokers n. (%) | 6 (14) |
| On-anti-hypertensive treatment n. (%) | 3 (7) |
| On anti-diabetics treatment n. (%) | 4 (9) |
| Systolic BP (mmHg) | 127±8 |
| Diastolic BP (mmHg) | 79±6 |
| Total cholesterol (mg/dL) | 199±40 |
| Triglycerides (mg/dl) | 121 (77-169) |
| Haemoglobin (g/dL) | 13.1±1.7 |
| Albumin (g/dL) | 4.4±0.2 |
| Glucose (mmol/L) | 99 (92-115) |
| Insulin (µUI/mL) | 37 (31-53) |
| HOMA-IR (µU/mL*mmol/L) | 1.26±0.8 |
| Azotemia (mg/dl) | 33±7 |
| Uric acid (mg/dl) | 5.6±1.6 |
| Total Bilirubin (mg/dl) | 0.4 (0.3-0.7) |
| GOT (UI/L) | 16.5 (12.2-25.7) |
| GPT (UI/L) | 30.0 (21.2-44.0) |
| CRP (mg/L) | 6.4 (3.3-15.6) |
| Creatinine (mg/dl) | 0.69±0.15 |

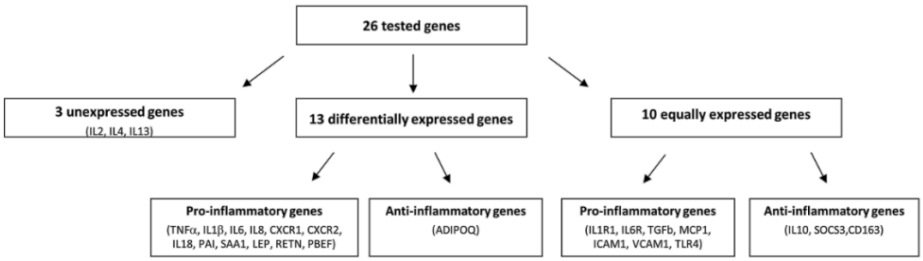
Data are expressed as mean± SD, median and inter-quartile range or as percent frequency, as appropriate

***Preliminary gene expression analysis in pooled samples***

From a total number of 26 cytokines tested in pooled samples (**Figure 1**), 13 genes including 12 pro-inflammatory genes (IL6, IL8, CXCR1, CXCR2, TNF$\alpha$, IL1$\beta$, IL18, PAI,

SAA1, visfatin, resistin, leptin) and just one anti-inflammatory gene (adiponectin) resulted to be differentially expressed in SAT compared with VAT. Ten genes were equally expressed (IL1R1, IL6R, IL10, TGFβ, MCP1, ICAM1, VCAM1, TLR4, SOCS3, CD163) in SAT and VAT and 3 anti-inflammatory genes were unexpressed (IL2, IL4 and IL13) in both fat compartments (**Figure 1**).

**Figure 1.** Flowchart representing the process for analyzing pro and anti-inflammatory gene expression in paired samples of SAT and VAT from 44 severely obese individuals




List of adipose tissue pro and anti-inflammatory genes tested

*Pro-inflammatory genes (n=19)*

IL1β: Interleukin-1 beta; IL1R1: Interleukin 1 receptor; TNFα: Tumor necrosis factor alpha; IL6: Interleukin 6; IL6R: Interleukin 6 receptor; TGFβ: Transforming growth factor beta; IL8: Interleukin 8; CXCR1: Interleukin 8 receptor 1; CXCR2: Interleukin 8 receptor 2; IL18: Interleukin 18; MCP1: Monocyte chemoattractant protein 1; SAA1: Serum amyloid A1; TLR4: Toll-like receptor 4; ICAM1: Intercellular adhesion molecule 1; VCAM1: Vascular cell adhesion molecule 1; PAI: Plasminogen activator inhibitor; LEP: Leptin; RETN: Resistin; PBEF: Pre-B cell colony-enhancing factor/visfatin

*Anti-inflammatory genes (n=7)*

IL2: Interleukin 2; IL4: Interleukin 4; IL10: Interleukin 10; IL13: Interleukin 13; SOCS3: Suppressor of cytokine signaling 3; CD163: Cluster of differentiation 163; ADIPOQ: Adiponectin

### *Gene expression analysis at individual level*

On the basis of findings in pooled samples, we undertook detailed analyses in individual patients.

Among the 13 differentially expressed genes, adiponectin, leptin, resistin and visfatin gene expressions were from 1.6 to 5.1 fold higher in SAT than in VAT (**Table 2**) and this was also true for all, but one inflammatory cytokine (IL18) which, instead, was 12 fold more expressed in VAT (**Table 2**).

**Table 2.** Gene expression measurements of pro and anti-inflammatory cytokines in paired samples of SAT and VAT

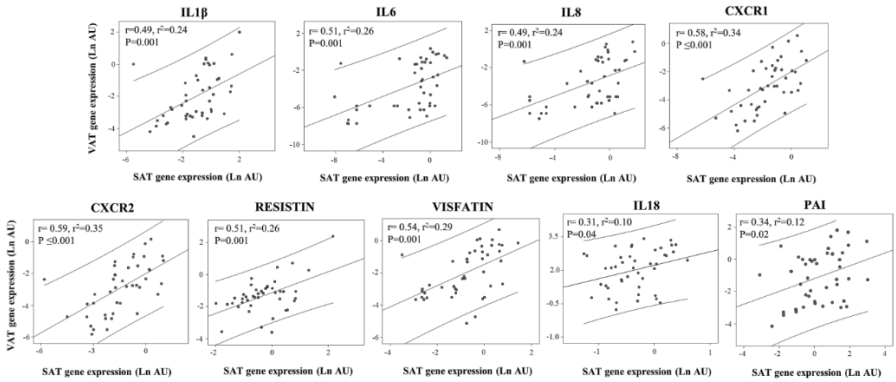
| Gene | Symbol | SAT median (IQR) | VAT median (IQR) | SAT/VAT | P value |
|---|---|---|---|---|---|
| *Pro-inflammatory genes* | | | | | |
| Tumor necrosis factor α | TNF α | 1.05 (0.71-1.57) | 0.64 (0.36-1.20) | 1.6 | 0.006 |
| Interleukin1 β | IL 1β | 0.46 (0.16-0.97) | 0.09 (0.04-0.41) | 5.1 | 0.001 |
| Interleukin 6 | IL6 | 0.48 (0.06-1.00) | 0.02 (0.003-0.325) | 19.2 | <0.001 |
| Interleukin 8 | IL8 | 0.51 (0.14-0.99) | 0.02 (0.003-0.305) | 20.4 | <0.001 |
| Interleukin 8 receptor, type 1 | CXCR1 | 0.23 (0.07-0.63) | 0.05 (0.01-0.16) | 4.6 | <0.001 |
| Interleukin 8 receptor, type 2 | CXCR2 | 0.21 (0.10-0.83) | 0.06 (0.01-0.20) | 3.5 | <0.001 |
| Interleukin 18 | IL18 | 0.69 (0.50-1.03) | 8.40 (3.50-15.20) | 0.08 | <0.001 |
| Serum amyloid A1 | SAA1 | 1.00 (0.70-1.54) | 0.63 (0.34-1.09) | 1.6 | 0.03 |
| Plasminogen Activator Inhibitor | PAI | 1.07 (0.51-3.97) | 0.31 (0.07-1.12) | 3.5 | <0.001 |
| Leptin | LEP | 0.77 (0.60-1.00) | 0.24 (0.10-0.42) | 3.2 | <0.001 |
| Resistin | RETN | 0.71 (0.43-1.30) | 0.25 (0.16-0.42) | 2.8 | <0.001 |
| Visfatin | PBEF | 0.51 (0.25-1.11) | 0.10 (0.04-0.52) | 5.1 | <0.001 |
| *Anti-inflammatory genes* | | | | | |
| Adiponectin | ADIPOQ | 2.02 (1.19-3.13) | 0.96 (0.58-1.76) | 2.1 | 0.001 |

Gene expression measurements (3rd and 4th columns) are expressed as arbitrary units and reported as median (inter-quartile range). The SAT/VAT ratio (5th column) represents the fold difference in cytokine gene expression measurements between SAT and VAT. In the last column the P value (Wilcoxon rank-sum test) of the difference between SAT and VAT gene expression measurements is also given.

The expression of IL6 and IL8 genes was about 20 times higher in SAT than in VAT. Of note, the expression level of seven pro-inflammatory genes (IL1β, IL6, IL8, CXCR1, CXCR2, resistin and visfatin) was strongly related ($r^2$ ranging from 0.24 to 0.35 and

P≤0.001) in SAT and VAT while the remaining two genes (IL18 and PAI) showed much weaker associations ($r^2$ =0.10 and $r^2$ =0.12, respectively) (**Figure 2**).

**Figure 2.** Correlation between cytokines gene expression in SAT and VAT. Gene expression measurements are expressed as arbitrary, Log-transformed units (Ln AU). Data are Pearson correlation coefficients (r), $r^2$ and P value



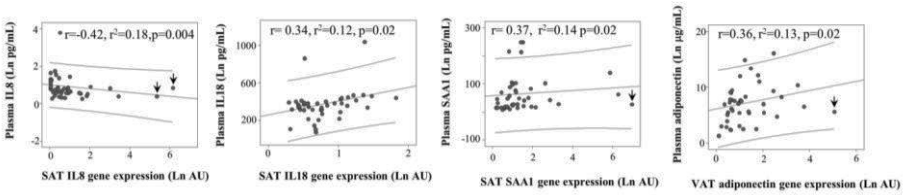

No relationship was found between SAT and VAT gene expression for the remaining 4 genes (adiponectin, leptin, TNFα and SAA1) (P≥0.20). A separate analysis by gender fully confirmed these results (data not shown).

***Functional link between expression of pro- and anti-inflammatory genes and circulating molecules***

Eleven gene products (TNFα, IL1β, IL6, IL8, IL18, SAA1, PAI, leptin, adiponectin, resistin, visfatin) were measured in plasma. Among these, only four inflammatory cytokines (IL8, IL18, SAA1, adiponectin) correlated with the corresponding gene expression in SAT or VAT. Plasma IL18 and SAA1 were directly related to the corresponding SAT gene expression while IL8 associated inversely with the corresponding gene expression (**Figure 3**). No relationship was observed between plasma levels of these three pro-inflammatory molecules and VAT gene expression of the corresponding genes (**Figure 3**). Among anti-inflammatory cytokines, only

adiponectin gene expression in VAT showed an association with the corresponding gene product levels in plasma (**Figure 3**).

**Figure 3.** Correlation between plasma levels of cytokines and the corresponding gene expression measurements in SAT or VAT. The arrows indicate the outliers identified by Mahalanobis distance test (see Ref. 14). The strength of these associations did not materially change after the inclusion of the outliers (all P≤0.02)




***Immune cells counting in SAT and VAT***

Given the specific involvement of immune cells in obesity-related inflammation, in a subgroup of 14 patients we counted CD163+ macrophages (a subpopulation of major relevance for the anti-inflammatory response) in SAT and VAT. We found that there was no difference in the number of CD163+ macrophages between SAT and VAT (3418±1353 n/cm$^2$ vs 3732±1396 n/cm$^2$, P=0.54), indicating that differences in the inflammatory status of the two fat compartments do not depend on the number of these cells.

**DISCUSSION**

In this study we quantified the gene expression of a large set of pro- and anti-inflammatory cytokines in abdominal SAT and VAT in severe obesity. The vast majority of pro-inflammatory genes were more expressed in SAT than in VAT whereas just one pro-inflammatory gene was more expressed in VAT, suggesting a stronger contribution of subcutaneous adipose compartment to the low-grade obesity-related inflammation.

***Gene expression in SAT and VAT***

Central adiposity is more strongly associated with adverse CV outcomes than peripheral adiposity (3). Although this risk excess is traditionally attributed to visceral fat (4), the predominant component of fat mass in central adiposity is subcutaneous rather than visceral (16).

We found that SAT and VAT express the same set of inflammatory cytokines in obese patients and that SAT, rather than VAT, is the fat compartment expressing the higher pro-inflammatory profile. This was true for fundamental fat cytokines like TNF$\alpha$ and IL6, the expression levels of these cytokines being from 1.6 to 20-fold greater in this fat compartment than in VAT (**Table 2**). These findings accord with previous studies focusing on TNF$\alpha$ and IL6 (10, 13, 17) and add weight to the contention that IL1$\beta$ gene expression is upregulated (10, 13), rather than downregulated (18), in SAT of obese patients. Furthermore, for the first time we show that IL8 is upregulated in SAT and that the gene expression of this chemokine is more than 20 times higher in SAT than in VAT. Such a remarkable increase of IL8 mRNA in SAT was paralleled by a significant increase of the gene expression of its corresponding receptors, i.e. CXCR1 and CXCR2, in the same fat compartment (**Table 2**), further suggesting an augmented role for IL8 signalling in SAT than in VAT in obese individuals. We also document an upregulated expression in SAT of other two important pro-inflammatory molecules like SAA1 (**Table 2**) which is involved in early response to injury, and PAI which is responsible for the negative regulation of the fibrinolytic system.

Leptin, Resistin and Visfatin are potent pro-inflammatory peptides. Consistently with previous studies (10, 12), we observed a 3-fold higher leptin and resistin gene expression in SAT. We found a similar pattern for visfatin, an insulin-mimetic peptide typically expressed in VAT. Of note, adiponectin, an anti-inflammatory cytokine, followed the same pattern, being twice more expressed in SAT than in VAT. IL18 was the sole pro-inflammatory cytokine showing a reverse expression pattern, being upregulated in the visceral rather than in the subcutaneous fat compartment. IL18 is a pleiotropic molecule promoting Th1 cell differentiation, cell-mediated cytotoxicity

and inflammation which also induces the synthesis of the anti-inflammatory cytokine IL10 (19) and limits the release of chemokines such as IL8 (20). IL18-deficiency in mice causes hyperphagia, obesity, diabetes and atherosclerosis by massive fat deposition in the arterial walls (21). Thus, IL18 downregulation in SAT is in keeping with the hypothesis that the overall expression profile of cytokines in SAT denotes a more pro-atherogenic, metabolically adverse attitude.

Although in our study the expression of pro-inflammatory genes was systematically upregulated in SAT, we found a strong and positive correlation between SAT and VAT gene expression for the majority of the pro-inflammatory genes studied (i.e. IL1β, IL6, IL18, IL8, CXCR1, CXCR2, resistin, visfatin and PAI) indicating that, though at different rates, the two main fat compartments undergo qualitatively similar changes in the expression profile of inflammatory cytokines.

Monocytes/macrophages typically accumulate in adipose tissue and are primarily responsible for the release of inflammatory mediators in this tissue (13, 22). Macrophage infiltration is of comparable extent in SAT and VAT (23) and we show that the number of M2 macrophages, i.e. CD163+ macrophages with anti-inflammatory potential, is identical in SAT and in VAT suggesting that in obesity a higher transcriptional activity rather than an expansion of M2 macrophages pool explains the increased pro-inflammatory gene expression of subcutaneous fat compartment.

***Gene expression profile and circulating gene products***

Circulating levels of inflammatory cytokines like TNFα (24), IL6 (25), IL8 (26), IL1β (27), IL18 (28), SAA1 (25), leptin (29) and resistin (30) are potent predictors of adverse cardiovascular outcomes. Adipose tissue cytokines mainly act as autacoids in the fat compartment. Interestingly, we found a strong association between adipose tissue gene expression and the corresponding plasma levels for 4 inflammatory cytokines (IL8, IL18, SAA1, adiponectin). Specifically, we found a positive correlation between SAT gene expression and plasma levels of IL18 and SAA1 and an inverse one in the same fat compartment between gene expression and plasma levels of IL8 (**Figure 3**). Adiponectin plasma levels were positively associated with adiponectin gene

expression only in VAT (**Figure 3**). Overall these findings provide circumstantial evidence that the adipose tissue *in vivo* may contribute to regulate circulating levels of, at least, some cytokines.

A potential limitation in our study is that because patients in this series had a low prevalence of diabetes and hypertension, selection bias cannot be excluded.

In conclusion, we show compartment-specific adipose tissue changes in inflammation-related genes in obesity and support the hypothesis that abdominal SAT contributes to the pro-inflammatory burden of severe obesity more than VAT, an observation also in keeping with the association of 3 pro-inflammatory cytokine genes with the corresponding gene product plasma levels. Whether the augmented pro-inflammatory profile of SAT in obese patients predicts CV events warrant further studies in this high risk population.

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## CHAPTER 3

# Tissue inhibitor of metalloproteinases (TIMP-1), genetic markers of insulin resistance and cardiomyopathy in patients with kidney failure

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**ABSTRACT**

**Background**: Left ventricular hypertrophy (LVH) is a major cardiovascular (CV) complication in patients with kidney failure and an association between polymorphisms in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene, a genetic marker of insulin resistance, and LVH and LV concentric remodelling has been recently documented in these patients.

**Aims**: Since myocardial fibrosis is a prominent feature in LVH induced by insulin resistance, we tested the hypothesis that the interaction between ENPP1 rs1974201 and rs9402349 polymorphisms and the Tissue Inhibitor of Metalloproteinases (TIMP-1) - a pro-fibrotic protein which inhibits extracellular matrix degradation - is implicated in concentric LVH and diastolic dysfunction in a cohort of 223 dialysis patients.

**Results**: Both ENPP1 polymorphisms rs1974201 and rs9402349 were in Hardy-Weinberg equilibrium in dialysis patients. In an analysis stratified by ENPP1 rs1974201 polymorphism, circulating levels of TIMP-1 in GG patients were coherently associated with two markers of concentric remodelling (RWT and LV mass to volume ratio) as well as with a marker of diastolic dysfunction (E/A ratio) (P ranging from 0.005 to 0.02) whereas no such associations existed in CC or CG patients. These observations suggest that the rs1974201 modifies the relationship between TIMP-1 and LV geometry and diastolic dysfunction. Accordingly, in a multiple regression model, an identical increase of TIMP-1 (100 ng/ml) was associated with an increase of 22% in RWT, 14% in LV mass to volume ratio and 29% in E/A ratio in GG patients but with almost no change (from -0.22 to 3.78%) in these echocardiographic indices in the remaining patients (P for the effect modification $\leq$0.024). The rs9402349 did not modify the relationship between TIMP-1 and LV geometry and function.

**Conclusion**: In dialysis patients, the ENPP1 rs1974201 polymorphism modifies the association between TIMP-1 and LV geometry and diastolic function. These results are consistent with the hypothesis that insulin resistance is involved not only in LVH but also in myocardial fibrosis, an alteration of primary importance in the high risk of this population.

**INTRODUCTION**

Left ventricular hypertrophy (LVH) is a pervasive complication of kidney failure and a strong predictor of death and adverse clinical outcomes in this population (1). From a structural point of view, cardiomyopathy in kidney failure is characterized by cardiomyocytes hypertrophy accompanied by prominent fibrosis. LV fibrosis depends on an altered balance between the accumulation and breakdown of cardiac extracellular matrix, a process regulated by matrix metalloproteinases (MMPs), a series of enzymes which are in turn inhibited by specific inhibitors [tissue inhibitors of metalloproteinases (TIMPs)]. Over-expression of TIMP-1 was observed in parallel with an increased LV mass in experimental models of pressure overload (2) and circulating levels of TIMP-1 were associated with LVH and LV diastolic impairment in individuals in the general population in the Framingham heart study (3) and in hypertensive patients as well (4-6). TIMP-1 is currently considered as a promising marker of myocardial fibrosis in cardiomyopathies (7, 8).

The ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene is a well characterized genetic marker of insulin resistance since it codes for a membrane glycoprotein that inhibits insulin receptor autophosphorylation thus altering the intracellular insulin signalling (9). In a recent study, we have found that two polymorphisms (i.e. rs1974201 and rs9402349) in the ENPP1 gene are associated with myocardial hypertrophy and LV concentric remodelling in dialysis patients (10). Since there is coherent evidence that insulin resistance promotes myocardial fibrosis (11), we investigated the hypothesis that genetic markers of insulin resistance in this population modify the link between a prototypic, pro-fibrotic cytokine at myocardial level like TIMP-1 and LV mass and function. To this scope, we sought whether ENPP1 gene and TIMP-1 levels interact in determining LV geometry and function in the same set of patients with kidney failure in which we described the association between the ENPP1 gene polymorphisms and LVH (10).

## SUBJECTS AND METHODS

The study protocol was in conformity with the ethical guidelines of our institution and informed consent was obtained by each participant.

### *Patients*

We studied an incident-prevalent cohort of 223 dialysis patients (125 males and 98 females, all Caucasian) who had been on regular dialysis treatment for at least 6 months, with left ventricular ejection fraction more than 35% and without cardiac circulatory congestion, major infections (fever, infected vascular access or peritonitis or exit site infection) or inter-current illnesses requiring hospitalization. One hundred and seventy-nine haemodialysis patients were being treated thrice weekly with standard bicarbonate dialysis (Na 138 mmol/L, HCO3 5 mmol/L, K 1.5 mmol/L, Ca 1.25 mmol/L, Mg 0.75 mmol/L) either with Cuprophan or semi-synthetic membranes. The average urea Kt/V in these patients was 1.21+0.26. The remaining 44 patients were on chronic ambulatory peritoneal dialysis (CAPD) and the average weekly urea Kt/V was 1.67+0.32. Thirty-five patients were diabetics and 91 were habitual smokers (22±17 cigarettes/day). Ninety-five patients were treated with various anti-hypertensive drugs (74 on mono-therapy with angiotensin-converting enzyme inhibitors, AT-1 antagonists, calcium channel blockers, □- and β-blockers and the remaining 30 on double or triple therapy with various combinations of these drugs). One hundred and fifteen patients were on treatment with erythropoietin. The main clinical and biochemical characteristics of the study population are detailed in **Table 1**.

### *Genotyping of the ENPP1 rs1974201 and rs9402349 polymorphisms*

Allelic discrimination for the two single nucleotide polymorphisms (SNPs) of ENPP1 gene, described under identification number rs1974201 and rs9402349, were performed by validated TaqMan SNP Genotyping Assays provided by Applied Biosystems on an ABI PRISM 7900HT Fast Real-Time PCR System, according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA), as

previously reported (10). A random 10% of samples were independently repeated to confirm genotyping results and they were completely consistent.

***Laboratory measurements***

Blood sampling was performed after an overnight fast always during a mid-week non-dialysis day for haemodialysis patients and at empty abdomen for CAPD patients. Blood was drawn and put into tubes containing EDTA, and plasma supernatants were stored at –80°C until batch analyses. All analyses were done blinded to clinical information. Serum cholesterol, albumin, calcium, phosphate and haemoglobin measurements were made using standard methods in the routine clinical laboratory. Plasma total homocysteine, ADMA and high sensitivity C-Reactive Protein (CRP) were measured as previously reported (12). Circulating levels of TIMP-1 were measured by an ELISA with the use of a Quantikine kit (intra-assay CV: 4.4%; inter-assay CV: 4.2%; upper limit of the normal range: 304 ng/ml. R&D Systems Inc, MN, USA).

***Echocardiography***

These studies were performed in a non-dialysis day for hemodialysis patients or at empty abdomen for those on CAPD within 2 hours after blood sampling. Left ventricular mass (LVM) was calculated according to the Devereux formula and indexed to height$^{2.7}$ (LVMI), as detailed in a previous study (13). Left ventricular hypertrophy (LVH) was defined by a LVMI of over 47 g/m$^{2.7}$ in women or over 50 g/m$^{2.7}$ in men. Left ventricular end diastolic volume (LVEDV) was calculated by the standard formula [(1.047*LVEDD$^3$)/body surface area]. The relative wall thickness [RWT: 2*posterior wall thickness/left ventricular end diastolic diameter (LVEDD)] and the LV mass-to-volume ratio, a ratio specifically applied in patients with kidney failure (14), were calculated as indexes of left ventricular concentric geometry. Values indicative of concentric left ventricular geometry were established on the basis of age-specific reference standards according to RWT (15). The ratio between early (E) and late (atrial - A) ventricular filling velocity (E/A ratio) was considered as an index of left ventricular diastolic function.

***Statistical analysis***

Data were summarized as mean ± standard deviation (normally distributed data), median and inter-quartile range (non-normally distributed data) or as percent frequency and comparisons between to groups were made by T-test, Mann-Whitney U Test or Chi Square test, as appropriate.

The effect modification of ENPP1 rs1974201 and rs9402349 polymorphisms on the relationship between circulating levels of TIMP-1 and LV geometry was investigated by univariate and multivariate linear and logistic regression models. Into the final model, we included TIMP-1, ENPP1 polymorphism (rs1974201 and rs9402349) and their interaction term as well as all variables that were related (with $P<0.05$) to the exposures (TIMP-1 and ENPP1 polymorphisms) or to the study outcomes (RWT, LV mass-to-volume ratio and E/A ratio). By this strategy we constructed models of adequate statistical power (at least 10 patients for each variable into the models). The estimated raise in RWT, LV mass to volume ratio and E/A ratio corresponding to a fixed increase in TIMP-1 (100 ng/ml) was derived from crude and fully adjusted regression coefficients and expressed as percentage change (± standard error). In the logistic regression analysis, data were expressed as odds ratio (OR) and 95% confidence interval (CI) and P value.

Data analysis was performed by a standard statistical package (SPSS for Windows, Version 9.01, Chicago, Illinois, USA).

**RESULTS**

The ENPP1 rs1974201 and rs9402349 polymorphisms were not in linkage disequilibrium (D'=0.735, r2=0.203) and their genotypic distributions (CC: 13%; CG: 42%; GG: 45% and AA: 71%; AC: 27%; CC: 2%) did not deviate from Hardy-Weinberg equilibrium ($\chi^2$=1.25, P=0.26 and $\chi^2$=0.11, P=0.74, respectively).

***Demographic, clinical and biochemical characteristics of patients with kidney failure as characterized by the ENPP1 rs1974201 and rs9402349 polymorphisms***

The demographic and clinical characteristics of patients divided according to the ENPP1 rs1974201 genotypes are presented in **Table 1**.

**Table 1.** Main demographic, somatometric and clinical characteristics of the study population

| | **ENPP1 (rs1974201) polymorphism** | | |
|---|---|---|---|
| | **CC or GC** (n=123) | **GG** (n=100) | P |
| Age (years) | 59,4±15,8 | 60,9±14,7 | 0.47 |
| Male sex n. (%) | 74 (60.2) | 51 (51) | 0.17 |
| Smokers n. (%) | 52 (42.3) | 39 (39) | 0.62 |
| Diabetics n. (%) | 16 (13) | 19 (19) | 0.22 |
| On anti-hypertensive treatment n. (%) | 46 (37.4) | 49(49) | 0.08 |
| Dialysis vintage (months) | 76,4±72,3 | 57,8±58,2 | 0.04 |
| With CV comorbidities n. (%) | 59 (48) | 47(47) | 0.89 |
| Systolic pressure (mmHg) | 130,7±22,5 | 136,9±21,7 | 0.04 |
| Diastolic pressure (mmHg) | 74,6±12,5 | 75,8±11,9 | 0.47 |
| Heart rate (beats/min) | 79,9±13,2 | 82,3±10,9 | 0.17 |
| BMI ($kg/m^2$) | 25,2±3,9 | 24,9±5 | 0.59 |
| Cholesterol (mg/dL) | 205,6±53,4 | 211,4±57,3 | 0.44 |
| Haemoglobin (g/L) | 10,9±1,8 | 10,5±2 | 0.10 |
| Albumin (g/L) | 4±0,5 | 4±0,6 | 0.55 |
| Calcium *Phosphate ($mMol^2/L^2$) | 4,4±1,1 | 4,5±1,3 | 0.52 |
| CRP (mg/L) | 6,7 (3,4-16) | 10,4 (1,7-4,3) | 0,04 |
| ADMA (μMol/L) | 2,8 (1,5-4,4) | 3,06 (1,8-4,3) | 0,67 |
| Homocysteine (μMol/L) | 27,2 (20-38,1) | 25,9 (20,2-46,47) | 0,96 |
| TIMP-1 (ng/ml) | 183,3 (158,7-214,7) | 187,3 (168,8-217,8) | 0,42 |

Data are expressed as mean± SD, median and inter-quartile range or as percent frequency, as appropriate. Comparisons among the two groups were made by t test (continuous variables) or Chi-Square Test (dichotomic variables) and non parametric U di Mann-Whitney test

GG homozygotes for this polymorphism had higher C reactive protein and systolic pressure and displayed a shorter dialysis vintage as compared to the group combining CG and CC genotypes. Moreover, GG patients tended to be more frequently treated with anti-hypertensive drugs (P=0.08). No differences were observed as for the remaining demographic, clinical or biochemical data (**Table 1**). The same analysis carried out for the rs9402349 polymorphism showed that AA patients had higher

serum cholesterol (212±58 mg/dL versus 198±48 mg/dL, P=0.03) and were more frequently on anti-hypertensive treatment (47% versus 32%, P=0.024) as compared to the group combining AC and CC patients. The two groups did not significantly differ as for the remaining clinical or biochemical data.

***TIMP-1, ENPP1 rs1974201 and rs9402349 polymorphisms and echocardiographic indicators of LV remodeling***

One hundred and eighty-seven patients out of 223 (84%) displayed LVH at echocardiography. Concentric LV geometry was the most frequent pattern (45%) (concentric LVH in 85 cases and concentric LV remodeling in 15 cases) followed by eccentric LVH (n=87) (39%). In the whole study population, TIMP-1 was directly associated to E/A ratio (r=0.17, P=0.014) but unrelated to RWT and LV mass-to-volume ratio (r ranging from 0.098 to 0.116, P=NS). A separate analysis by ENPP1 rs1974201 genotypes, showed that in GG patients, circulating levels of TIMP-1 directly related to RWT (r=0.25, P=0.01), LV mass-to-volume ratio (r=0.24, P=0.02) and E/A ratio (r=0.28, P=0.005) while no such an association existed in patients with CG or CC genotypes. In a similar analysis by rs9402349 genotypes, TIMP-1 correlated with E/A ratio (r=0.19, P=0.02) in AA patients while no other relationships were found between this biomarker and the other echocardiographic indices (P=NS).

***Effect modification by ENPP1 rs1974201 polymorphism of TIMP-1-LV geometry relationship***

On crude analysis, the ENPP1 rs1974201 polymorphism modified the relationship between circulating levels of TIMP-1 and echocardiographic indicators of LV remodeling and E/A ratio (**Fig. 1**).

Indeed, in a regression analysis stratified according to genotypes, a 100 ng/ml increase in circulating levels of TIMP-1 was associated with a 22% increase in RWT, 14% in LV mass to volume ratio and 29% in E/A ratio in patients with GG genotype but with minor or no change in patients with CC or CG genotypes (RWT: -0.2%; LV mass to volume ratio: -1.0%; E/A ratio: +3.8%) (**Table 2**). Data adjustment for potential confounders did not modify these relationships (**Table 2 - Adjusted models**).

**Figure 1.** Correlation analyses between circulating levels of TIMP-1 with RWT, LV mass-to-volume ratio and E/A ratio separately in CC or CG patients versus GG patients. Data are Pearson product moment correlation coefficient (r) and P value. At the bottom of each couple of graphs, the P value for the interaction (or effect modification) is reported.

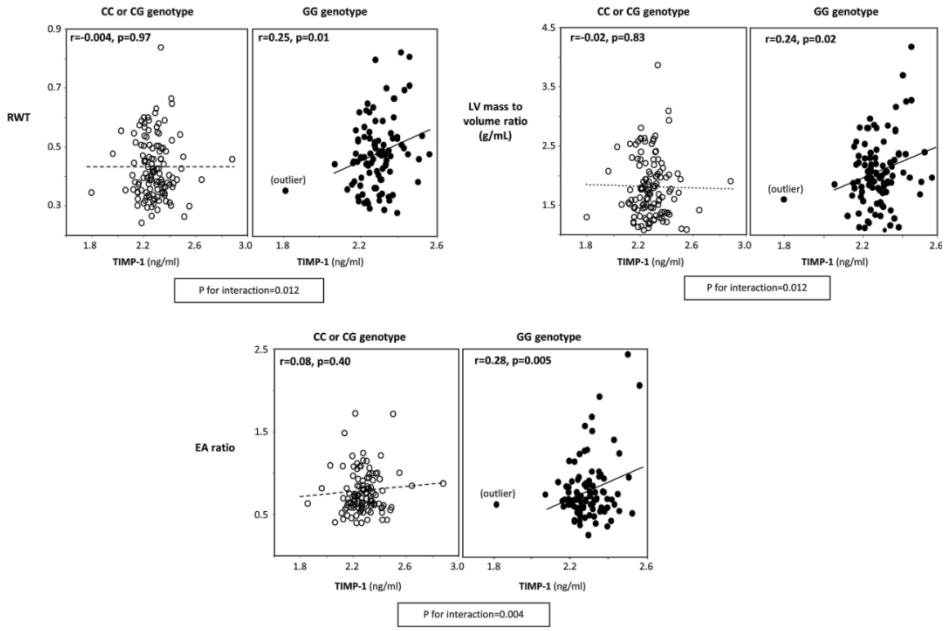



**Table 2.** Multiple linear regression analyses of the interaction between TIMP-1 and ENPP1 rs1974201 for explaining RWT, LV mass to volume ratio and EA ratio.

| | | **Regression coefficients (% change), standard errors and P values** | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Crude* | | | *Adjusted** | | |
| | | **CC+CG** | **GG** | **P** | **CC+CG** | **GG** | **P** |
| **TIMP-1** (100 ng/mL increase) versus | **RWT** | -0.2±2.2 | 22.1±2.2 | **0.012** | -0.7±2.2 | 22.1±2.2 | **0.019** |
| | **LV mass to volume ratio** | -1.0±3.6 | 14.5±3.6 | **0.012** | -1.6±3.1 | 14.0±3.1 | **0.024** |
| | **EA ratio** | 3.8±5.0 | 29.0±5.0 | **0.004** | 3.8±5.0 | 29.0±5.0 | **0.002** |

***** Model adjusted for: systolic blood pressure, age, anti-hypertensive treatment, smoking, ADMA, CRP and albumin (see Methods-Statistical Analysis for more details)

Forcing diabetes, gender and CV comorbidities into the multivariate analyses, these covariates did not affect the strength of the relationships between TIMP-1 and RWT, LV mass to volume ratio and E/A ratio (data not shown). When a similar analysis was carried out for the ENPP1 rs9402349, the genotypes of this polymorphism did not significantly affect the association between TIMP-1 and the echocardiographic parameters (P=NS).

***ENPP1 rs1974201 polymorphism and TIMP-1 and the risk for LV concentric remodeling***

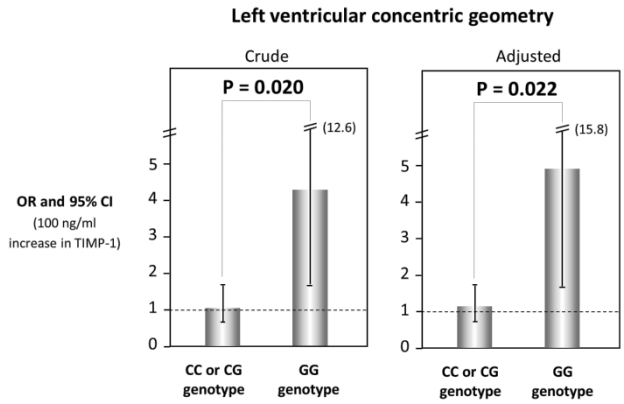
The prevalence of concentric LV geometry was significantly higher (P=0.006) in GG (55%) than in GC and CC patients (45%). Both on crude and fully adjusted logistic regression analyses (**Table 3**), an identical increase in circulating levels of TIMP-1 (100 ng/ml) was associated with a higher risk for concentric LV geometry (P=0.02) in GG than in CG and CC patients (**Fig. 2**) and this was also true for concentric LV hypertrophy [**GG patients**: adjusted OR (100 ng/ml increase in TIMP-1): 4.07, 95% CI: 1.32-12.52; **CG and CC patients**: adjusted OR (100 ng/ml increase TIMP-1): 1.09, 95% CI: 0.64-1.88) (P for the effect modification=0.039).

**Table 3.** Multiple logistic regression analysis of the interaction between TIMP-1 and ENPP1 rs1974201 for explaining concentric left ventricular geometry

| **Variables** | **Left ventricular concentric geometry** | |
|---|---|---|
| | **Odds ratio (95% CI) and P** | |
| | *Crude* | *Adjusted* |
| TIMP-1 (*100 ng/mL increase*)<br>ENPP1 rs1974201 (*CC+CG=0; GG=1*)<br>TIMP-1 (*100 ng/mL increase*)*ENPP1 rs1974201 (*CC+CG=0; GG=1*) interaction term | P for interaction=0.02<br>**(see Fig.2)** | P for interaction= 0.022<br>**(see Fig.2)** |
| Systolic blood pressure (1 mmHg)<br>Age (1 year)<br>Anti-hypertensive treatment (0=no; 1=yes)<br>Smoking (0=no; 1=yes)<br>ADMA (1µmol/L)<br>CRP (1 mg/L)<br>Albumin (1 g/dL) | | 1.00(0.99-1.02), P=0.73<br>0.99(0.97-1.02), P=0.59<br>1.80(0.96-3.50), P=0.07<br>1.05(0.57-1.91), P=0.87<br>1.22(1.06-1.41), P=0.005<br>0.99(0.98-1.01), P=0.61<br>0.98(0.92-1.03), P=0.48 |

Data are expressed as odds ratio, 95% confidence intervals and P value.

**Figure 2.** Effect modification of ENPP1 rs1974201 polymorphism on the crude and adjusted odds ratio for left ventricular concentric geometry corresponding to a fixed increase in TIMP-1 (100 ng/ml) in patients with and without GG genotype. Data are expressed as odds ratio and 95% CI and P value.




**DISCUSSION**

This study shows that in dialysis patients the rs1974201 polymorphism in the ENPP1 gene, a genetic marker of insulin resistance in this population, modifies the relationship between TIMP-1 and left ventricular geometry and diastolic function. These findings are in line with the hypothesis that fibrosis is an important component in LV remodeling and hypertrophy triggered by insulin resistance in this population.

In close parallelism with studies in the remnant kidney model (16), postmortem studies in patients with kidney failure have coherently shown that LVH and structural remodeling of myocardium is characterized by hypertrophy of cardiomyocytes accompanied by an abnormal accumulation of fibrous tissue in the interstitium of the myocardium (17). Furthermore, ultrasonic myocardial characterization studies *in vivo* in patients with kidney failure (18, 19) confirmed that fibrosis is a hallmark in LVH in dialysis patients. Myocardial fibrosis, in the setting of LVH, is not unique to kidney failure and may occur in several conditions including hypertension (20), hyperaldosteronism (21, 22) and hyperinsulinemia and insulin resistance (23). As a

matter of fact, insulin sensitivity as measured by whole body glucose disposal explains the 19% of the variability in the muscular component of the LV in essential hypertensives (24). The causal implication of insulin resistance in LVH in these patients is also supported by the observation that regression of myocardial hypertrophy goes strictly along with improvement in insulin resistance in longitudinal studies (25). Excess of collagen at myocardial level results from a derangement in the dynamic balance between collagen synthesis by cardiac fibroblasts and collagen degradation by matrix metalloproteinases (MMPs). The proteolytic activity of MMPs is regulated by tissue inhibitors of metalloproteinases (TIMPs), particularly by TIMP-1 (26), and an unbalanced relationship between TIMP-1 and MMPs results in myocardial fibrosis. Indeed, in spontaneously hypertensive rats (SHRs), increased myocardial expression of TIMP-1 largely explains the diminished collagenase activity and excess fibrosis in this experimental model (2). Coherently with animal studies, it was demonstrated that plasma TIMP-1 is a marker of LVH and myocardial fibrosis both in individuals in the general population (3) and in essential hypertensives (4-6). Of note, MMP/TIMP balance is affected by insulin because this hormone shifts this balance toward reduction of extracellular matrix degradation via the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase Akt pathway (27, 28).

We have recently described that two polymorphisms (i.e. rs1974201 and rs9402349) in the ENPP1 gene which associate with myocardial hypertrophy and LV concentric remodeling in dialysis patients (10). Of note, in that study the rs1974201 polymorphism, but not the rs9402349 polymorphism, showed also a parallel association with insulin and glucose levels (10). Because excess of fibrosis is a hallmark of concentric LVH in kidney failure, it appears possible that gene polymorphisms conducive to insulin resistance also modify the link between TIMP-1, a pro-hypertrophic and pro-fibrotic compound, and LVH geometry and function. In line with this hypothesis, we found that ESRD patients homozygous for the G allele of the ENPP1 rs1974201 polymorphism - precisely the same polymorphism showing a link with LVMI and insulin levels as well (10) - exhibited a direct association between TIMP-1 and the

echocardiographic indices of LV geometry (RWT, LV mass-to-volume ratio) as well as with a fundamental marker of diastolic dysfunction like the E/A ratio. Thus, the ENPP1 rs1974201 predisposes not only to concentric remodeling but also to a pro-fibrotic response to TIMP-1. The functional relevance of this relationship is underlined by the inverse association between TIMP-1 and E/A ratio in GG homozygous patients implying progressively more severe diastolic dysfunction at increasing TIMP-1 levels in these patients. These findings may have clinical implications because altered diastolic function is a death predictor in kidney failure (29, 30).

Our study is limited in many respects. The first and the most obvious limitation is sample size. To avoid reporting false positive associations, large sample size and confirmatory analyses in diverse populations are recommended for testing genetic effects. However, it is important noting that the hypothesis we probed here was tested in an ethnically homogeneous cohort and had a strong “a priori” based on the previous association between concentric LV remodeling and ENPP1 gene variants in the same cohort (10). Furthermore, our study had also a precise biological rationale because insulin reduces collagen degradation by affecting the MMP/TIMP balance (26). Second, the cross-sectional design of the study precludes the possibility to draw definitive conclusions about the nature (causal/not causal) of the relationships we found. Third, we chose to measure plasma TIMP-1 because experimental data support a more important role of this enzyme compared to other tissue inhibitors of metalloproteinases, but we measured neither other TIMPs nor metalloproteinases.

In conclusion, in a sizable series of dialysis patients, we show that the genetic variability in the ENPP1 gene, a major modulator of insulin sensitivity, modifies the relationship between the pro-fibrotic enzyme TIMP-1 and LV geometry and function. These data further support the concept that ENPP1 gene is a relevant player in the pathogenesis of concentric LVH and myocardial fibrosis in patients with kidney failure.

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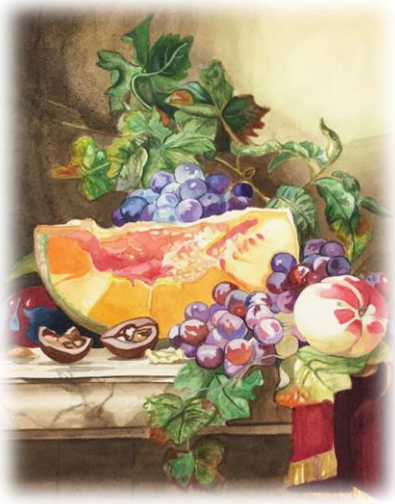
# PART II

## Insulin resistance, inflammation and oxidative stress in obesity and life expectancy: prospective data from kidney disease and elderly patients



## CHAPTER 4

# Association of IL-6 and a functional polymorphism in the IL-6 gene with cardiovascular events in patients with CKD

Spoto B, Mattace-Raso F, Sijbrands E, Leonardis D, Testa A, Pisano A, Pizzini P, Cutrupi S, Parlongo RM, D'Arrigo G, Tripepi G, Mallamaci F, Zoccali C.

*Clin J Am Soc Nephrol. 2015;10:232-40*

**ABSTRACT**

**Background and objectives**: High serum interleukin-6 (IL-6) is a major risk factor for cardiovascular disease (CVD) in the general population. This cytokine is substantially increased in chronic kidney disease (CKD) patients but it is still unknown whether the link between IL-6 and CVD in CKD is causal in nature.

**Design, setting, participants and measurements**: In a cohort of 755 stage 2-5 CKD patients, consecutively recruited from 22 Nephrology Units in Southern Italy, we studied the relationship of serum IL-6 with history of CVD as well as with incident cardiovascular (CV) events (follow up: 31±10 months) and used the functional polymorphism (-174 G/C) in the promoter of the IL-6 gene to investigate whether the IL-6-CV events link is causal in nature.

**Results**: In adjusted analyses, serum IL-6 above the median value was associated with history of CVD (P<0.001) and predicted the incidence rate of CV events (HR:1.66, 95%CI:1.11-2.49; P=0.01).

Patients homozygous for the risk allele (C) of the -174 G/C polymorphism had higher levels of IL-6 than those with other genotypes (P=0.04). Homozygous CC patients had more frequently history of CVD (OR:2.15, 95%CI:1.15-4.00; P=0.02) as well as a 87% higher rate of incident CV events (HR:1.87, 95%CI:1.02-3.44; P= 0.04) as compared to other genotypes.

**Conclusions**: In stage 2-5 CKD patients, high serum IL-6 is associated with history of CVD and predicts incident CV events. The parallel relationship with history of CVD and incident CV events of the -174 G/C polymorphism in the IL-6 gene suggests that IL-6 may be causally involved in the high CV risk in this population.

**INTRODUCTION**

Classical experimental studies by Russel Ross et al. in the nineties solidly established inflammation as a critical component of the atherosclerosis process (1). Over the last two decades, large cohort studies in the general population have shown strong links between biomarkers of inflammation and cardiovascular (CV) outcomes in the general population (2-5) and in patients with cardiovascular disease (CVD) (6, 7). Observational studies are methodologically vulnerable to test causality because these studies are open to various sources of bias and confounding. Mendelian randomization – i.e. the random assortment of alleles at conception – offers an intriguing opportunity to limit problems inherent to observational studies because categorization of patients according to pertinent alleles is a sort of genetic randomization. Genetic variants may thus be used as indicators of environmental exposures in the observational context (8). Large scale Mendelian randomization studies applying genetic polymorphisms of inflammatory cytokines (9) offer strong support to the hypothesis that, like in experimental animals (10, 11), the link between inflammation and atherosclerosis complication is causal in nature.

Cardiovascular risk is a multifactorial problem in CKD patients (12). Systemic inflammation is common in CKD patients, particularly in stage 5 CKD patients on dialysis (13, 14). In line with studies in the general population (5, 15), IL-6, a major pro-inflammatory cytokine, is an established strong predictor of adverse clinical outcomes in stage 5D CKD patients (16-20). However, the relationship between IL-6 and CV disease at earlier CKD stages was investigated in just one relatively small study by Barreto et al. (21) which was based on a limited number of CV events (just 22 events). In addition, to date we lack specific proof that this relationship in the CKD population is causal in nature. Circulating levels of IL-6 are genetically regulated. Since transmission of genes is a random phenomenon, gene polymorphisms modulating IL-6 synthesis may represent an unbiased means for testing whether the link between IL-6 and CV outcomes in CKD patients is causal in nature (Mendelian randomization). The -174 G/C single nucleotide polymorphism is a

functional variant located in the promoter region of the IL-6 gene which regulates the rate of IL-6 gene transcription (22-28) and therefore represents a reliable research tool for testing the nature (causal *vs* non causal) of the link between IL-6 and CV outcomes in CKD. With this background in mind, we set out to confirm findings by Barreto et al. (21) in a large observational study including a carefully characterized cohort of 755 stage 2-5 CKD patients and to test whether this relationship may underlie a causal link by applying the Mendelian Randomization approach i.e. by stratifying the study population according to the functional -174 G/C polymorphism in the IL-6 gene.

## METHODS

### *Study protocol*

The study protocol was in conformity with the ethical guidelines of our institution and it was approved by ethical committees of all participating Units. Written informed consent was obtained from each participant.

### *CKD patients*

Our study population included a genetically homogenous series of 755 Caucasian patients belonging to the same geographical area (Southern Italy) (29), consecutively recruited from 22 Nephrology Units in a period extending from October 2005 to September 2008. Eligible patients were age 18 to 75 years and in stable clinical condition. Exclusion criteria included acute or rapidly evolving renal disease, kidney transplant, acute inter-current infections or acute inflammatory processes, pregnancy, cancer or diseases in the terminal phase. This cohort was described in detail elsewhere (30).

### *Control population*

To compare the allelic frequencies of the -174 G/C polymorphism observed in CKD patients, we studied a sample of 463 consecutive blood donors of the general population from the same geographical area of CKD patients.

***Follow-up and study outcome***

After the initial assessment, patients were monitored for 31±10 months (range: 0.3 to 48 months). The study end-point was fatal and non-fatal cardiovascular events as described elsewhere (30). These events included myocardial infarction documented by ECG and biomarkers of myocardial injury; heart failure defined as dyspnea in addition to two of the following conditions: raised jugular pressure, bi-basilar crackles, pulmonary venous hypertension or interstitial edema on chest x-ray requiring hospitalization; ECG documented arrhythmia; stroke; peripheral vascular disease; major arterial or venous thrombotic episodes. These events were accurately recorded during the follow-up period.

The history of CVD was defined as the presence of at least one of the following comorbidities at enrolment: myocardial infarction, heart failure, peripheral vascular disease, stroke, transient ischemic attack or coronary surgery/angioplasty.

***Laboratory measurements***

In the whole study population blood sampling was performed in the early morning after an overnight fast and plasma was stored at - 80°C until analysis. Serum glucose, lipids, hemoglobin, albumin, creatinine and C-reactive Protein (CRP) were measured by standard methods in the routine clinical laboratory. Serum IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., Minneapolis, USA). Estimated glomerular filtration rate (eGFR) was calculated by using the 4-variables MDRD study equation (31) and not by CKD-EPI formula since the creatinine data were not IDMS traceable during the study period. All CKD patients underwent a 24h urinary collection for the measurement of proteinuria.

***Genotyping of -174G/C polymorphism***

Allelic discrimination of -174 G/C polymorphism was performed using a custom TaqMan SNP Genotyping Assay provided by Applied Biosystems (Applied Biosystems, Foster City, CA, USA). In this assay, primers were designed to amplify a region including the mutation site specifically recognized by a couple of probes able to discriminate wild-type and mutated alleles. The sequences of primers and probes were: 5'-

CGACCTAAGCTGCACTTTTCC-3' (forward primer) and 5'-GGGCTGATTGGAAACCTTATTAAGATTG-3' (reverse primer); 5'-CCTTTAGCAT[G]GCAAGAC-3' (C allele-specific probe) and 5'-CCTTTAGCAT[C]GCAAGAC-3' (G allele-specific probe). Allelic discrimination was performed on a 7900HT Fast Real-Time PCR platform and its accompanying Sequence Detection System (SDS) Software version 2.4 (Applied Biosystems, Foster City, CA, USA). Briefly, genomic DNA was extracted from peripheral blood mononuclear cells using standard salting out procedure (32). The reaction system contained 20 ng of genomic DNA, 12.5 μl of 2 X TaqMan Universal PCR Master Mix No AmpErase UNG, 1,25 μl of 40 X Assay Mix (including unlabeled PCR primers, FAM and VIC dye-labeled TaqMan MGB probes) and $H_2O$ for a total volume of 25 μl. A random 10% of samples were independently repeated to confirm genotyping results. The genotype results for these samples were completely consistent. All analyses were done blinded to clinical information.

***Statistical analysis***

Data were expressed as mean ± standard deviation (SD), median and inter-quartile range (IQR) or as percent frequency and comparison between two groups were made by T Test, Mann-Whitney Test or Chi Square test, as appropriate. The comparison among more than two groups was performed by ANOVA for log transformed variables, when appropriate. The deviation from Hardy-Weinberg equilibrium was assessed by the Chi Square test comparing observed and expected genotype frequencies. The 95% confidence interval of the risk allele frequency was calculated as suggested by the standard method (33).

The functional form of serum IL6 (as continuous, binary, quartiles or quintiles data) was formally investigated by the analysis of Martingale residuals and the binary form (below/above the median) provided the best data fitting.

The relationships between serum levels of IL-6 and cardiovascular disease was performed by two approaches. First, we analyzed the baseline association between serum IL-6 with history of CVD. Second, we investigated the predictive power of serum

IL-6 in the prospective cohort study. In both logistic and survival analyses, we considered variables which met criteria to be confounders [i.e. variables related ($P \leq 0.10$) to both the exposure under investigation (serum IL-6 levels below/above the median value) and history of CVD or incident fatal and non-fatal CV events, which are not an effect of the exposure and are not in the causal pathway between the exposure and outcome)] (34). Tested covariates included traditional risk factors (age, gender, smoking, diabetes and glucose, cholesterol and blood pressure), factors peculiar to CKD (hemoglobin, albumin, eGFR and urinary protein), anti-hypertensive treatment, BMI and C-reactive protein. In both logistic and Cox regression models, eGFR was always forced because of the strong and significant correlation between eGFR and serum IL-6 (**Table 1a**). To further investigate the causal role of IL-6 in the pathway leading to CV events in CKD patients, we applied a Mendelian randomization approach, i.e. we stratified the study population according to the -174 G/C polymorphism. These analyses were appropriately adjusted for variables which differed between CC and GC or GG patients and which appeared to be potential confounders (i.e. age, gender, cholesterol) for the interpretation of the link between the risk genotype (CC) and the study outcomes.

In the prospective cohort study, the potential distortion on the study results due to the competing risks of death was assessed by comparing the incidence rate of death in exposed (high IL-6 levels or CC risk genotype) and unexposed patients (low IL-6 levels or GC/GG genotype). If a difference was found, the competing risk of death was accounted by carrying out a survival analysis considering a combined outcome "death/CV events" (35). Data were expressed as odds ratio (logistic regression analysis), hazard ratio (Cox regression model), 95% confidence interval (CI) and P value. To internally validate the independent relationships of serum IL-6 and -174 G/C polymorphisms with history of CVD and incident CV events, a bootstrap resampling technique of 1,000 samples (randomly extracted from the original sample) was performed (36). All potential effect modifications exerted by covariates on the relationship between the key exposures (serum IL-6 and -174 G/C polymorphism) and

study outcomes were formally tested by introducing a multiplicative term into the models and no significant interaction was found.

All calculations were made by using a standard statistical package (IBM SPSS Statistics for Windows, Version 21.0. 0.1, Armonk, NY: IBM Corp.).

**RESULTS**

We studied 755 patients with stages 2-5 CKD (stage 2: 3.0%; stage 3a: 22.0%; stage 3b: 38.0%; stage 4: 34%, stage 5: 3.0%). Four hundred and fifty-three patients were males (60%), 263 were patients with type-2 diabetes, 98 were current smokers (13%). Two-hundred and twenty-one patients (29%) had history of CVD (**Table 1a**). One hundred and nine had only one past CV event while 112 of them had two or more than two past CV events. The first CV event in this population was myocardial infarction in 19 cases, heart failure in 26 cases, peripheral vascular disease in 29 cases, stroke in 12 cases, transient ischemic attack in 12 cases and coronary surgery/angioplasty in 11 patients. Six hundred ninety-one (92%) were on antihypertensive treatment: 19% were treated with one medication, 29% with two medications, 28% with three medications and the 16% with four or more medications. Estimated GFR was on average 36±13 ml/min/1.73 $m^2$ and the median 24h urinary protein was 0.6 mg/24h (inter-quartile range: 0.2-1.5 mg/24h). The median IL-6 was 2.5 pg/ml (inter-quartile range: 1.6-4.0 pg/ml).

***Analyses based on serum IL-6 levels***

Patients with IL-6 above the median value were significantly older, more frequently diabetics and had higher serum glucose and 24h urinary protein than those with IL-6 below this threshold **(Table 1)**. Estimated GFR, hemoglobin and albumin were lower in patients with higher IL-6 as compared with those with IL-6 below the median value. Systolic blood pressure (BP) was higher and diastolic BP lower in patients with IL-6 levels above the median **(Table 1)**.

**Table 1 a.** Main demographic and clinical characteristics of the study population according to serum IL-6 levels

| | **Whole group** (n=755) | **Serum IL-6 <2.5 pg/mL** (n=380) | **Serum IL-6 ≥2.5 pg/mL** (n=375) | **P** |
|---|---|---|---|---|
| Age (years) | 62±11 | 59±12 | 64±9 | <0.001 |
| Males (%) | 453 (60) | 224 (59) | 229 (61) | 0.55 |
| Diabetes (%) | 263 (35) | 105 (28) | 158 (42) | <0.001 |
| Current Smokers (%) | 98 (13) | 55 (14) | 43 (11) | 0.22 |
| CV comorbidities (%) | 221 (29) | 82 (22) | 139 (37) | <0.001 |
| BMI (kg/m$^2$) | 28.2±4.7 | 27.3±4.3 | 29.0±4.8 | <0.001 |
| Systolic BP (mmHg) | 134±18 | 132±17 | 135±19 | 0.03 |
| Diastolic BP (mmHg) | 78±11 | 79±10 | 77±11 | 0.01 |
| On anti-hypertensive treatment (%) | 691 (92) | 341 (90) | 350 (93) | 0.89 |
| Glucose (mg/dL) | 116±49 | 109±41 | 122±56 | <0.001 |
| Total cholesterol (mg/dL) | 187±45 | 189±43 | 184±46 | 0.12 |
| Hemoglobin (g/dL) | 12.8±1.8 | 13.1±1.8 | 12.6±1.8 | <0.001 |
| Albumin (g/dL) | 4.2±0.5 | 4.2±0.5 | 4.1±0.5 | 0.01 |
| CRP (mg/L) | 2.4 (1.0-5.5) | 1.4 (0.7-2.8) | 4.3 (1.9-9.0) | <0.001 |
| Phosphate (mg/dL) | 3.7±0.8 | 3.6±0.7 | 3.8±0.8 | 0.02 |
| eGFR (ml/min/1.73m$^2$) | 36±13 | 38±13 | 34±13 | <0.001 |
| Urinary protein (g/24h) | 0.6 (0.2-1.5) | 0.5 (0.2-1 .2) | 0.6 (0.2-1.7) | 0.01 |

Data are expressed as mean ±standard deviation, median and inter-quartile range and as percent frequency, as appropriate.
Abbreviations: BMI: body mass index, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate

As expected, serum CRP levels were directly associated with IL-6 levels (r=0.57, P<0.001). On bivariate, multivariate and bootstrapping validation analyses, patients with IL-6 above the median were more likely to have had a history of CVD (P≤0.01) than those with IL-6 below the median (**Table 2**).

**Table 2**. Logistic regression models of history of CVD

| | **Bivariate** | | **Multivariate** | | **Bootstrapping validation** | |
|---|---|---|---|---|---|---|
| | OR (CI 95%) | P | OR (CI 95%) | P | OR (CI 95%) | P |
| *Serum IL-6 [**0** (<2.5 pg/mL); **1**(≥2.5 pg/mL)] | 2.14 (1.55-2.95) | <0.001 | 1.58 (1.11-2.24) | 0.01 | 1.58 (1.12-2.24) | 0.01 |
| Age (years) | | | 1.05 (1.03-1.07) | <0.001 | 1.05 (1.03-1.07) | <0.001 |
| Diabetes (0=no; 1=yes) | | | 2.83 (1.99-4.03) | <0.001 | 2.83 (1.97-4.08) | <0.001 |
| Systolic BP (mmHg) | | | 1.00 (0.99-1.01) | 0.93 | 1.00 (0.99-1.01) | 0.94 |
| Haemoglobin (g/dL) | | | 1.04 (0.94-1.16) | 0.47 | 1.04 (0.93-1.16) | 0.49 |
| Albumin (g/dL) | | | 0.74 (0.50-1.11) | 0.15 | 0.74 (0.50-1.11) | 0.14 |
| Phosphate (mg/dL) | | | 1.02 (0.80-1.30) | 0.86 | 1.02 (0.78-1.34) | 0.87 |
| Urinary protein (mg/24h) | | | 1.01 (0.89-1.13) | 0.93 | 1.01 (0.88-1.14) | 0.93 |
| eGFR (ml/min/1.73m$^2$) | | | 1.00 (0.98-1.01) | 0.57 | 1.00 (0.98-1.01) | 0.57 |

*Below/above the median value (in parenthesis). The description of the model building strategy is reported in the Method section.

Abbreviations: eGFR: estimated glomerular filtration rate.

During the follow-up, 42 patients died. The incidence rate of mortality was 3 times higher (P=0.01) in patients with IL-6 above the median (3 deaths per 100 person-years, 95% CI: 2.0-4.1) than in those with IL-6 below this threshold (1 death per 100 person-years, 95% CI: 0.6-2.1). Overall, 117 patients had fatal and non-fatal CV events. As shown in **Table 3**, on bivariate, multivariate and bootstrapping validation analyses, the incidence rate of fatal and non-fatal CV outcomes was significantly higher (P≤0.02) in patients with IL-6 above the median (8.6 CV events per 100 person-years, 95% CI: 2.5-5.0) than in those with IL-6 below this threshold (3.6 CV events per 100 person-years, 95% CI: 6.9-10.8) (**Figure 1**, left panel).

**Table 3.** Cox regression models of incident CV events

| | **Bivariate** | | **Multivariate** | | **Bootstrapping validation** | |
|---|---|---|---|---|---|---|
| | HR (CI 95%) | P | HR (CI 95%) | P | HR (CI 95%) | P |
| *Serum IL-6 [**0** (<2.5 pg/mL); **1** (>2.5 pg/mL)] | 2.37 (1.61-3.51) | <0.001 | 1.66 (1.11-2.49) | 0.01 | 1.66 (1.10-2.52) | 0.02 |
| Age (years) | | | 1.07 (1.04-1.10) | <0.001 | 1.07 (1.03-1.10) | <0.001 |
| Diabetes (0=no; 1=yes) | | | 1.59 (1.09-2.32) | 0.02 | 1.59 (1.06-2.37) | 0.02 |
| Systolic BP (mmHg) | | | 1.00 (1.00-1.02) | 0.31 | 1.00 (1.00-1.02) | 0.31 |
| Haemoglobin (g/dL) | | | 0.91 (0.80-1.02) | 0.10 | 0.91 (0.81-1.02) | 0.09 |
| Albumin (g/dL) | | | 0.82 (0.53-1.27) | 0.38 | 0.82 (0.52-1.29) | 0.39 |
| Phosphate (mg/dL) | | | 1.13 (0.88-1.44) | 0.34 | 1.13 (0.88-1.45) | 0.35 |
| Urinary protein (mg/24h) | | | 1.05 (0.94-1.17) | 0.38 | 1.05 (0.93-1.18) | 0.44 |
| eGFR (ml/min/1.73m$^2$) | | | 1.01 (0.99-1.02) | 0.44 | 1.01 (0.99-1.02) | 0.49 |

*Below/above the median value (in parenthesis). The description of the model building strategy is reported in the Method section.

Abbreviation: eGFR: estimated glomerular filtration rate

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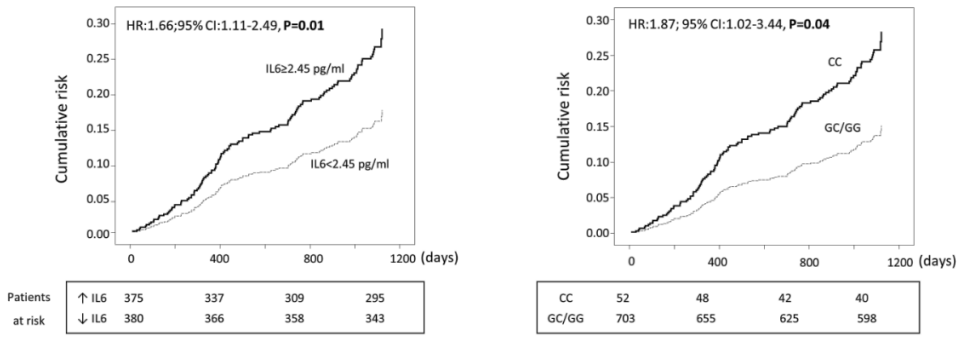


**Figure 1**. Serum levels of IL-6 (left panel) and C reactive protein (right panel) according to -174 G/C polymorphism in IL-6 gene. Data are expressed as medians. The inter-quartile ranges are indicated in parentheses. The comparison among groups was made by ANOVA

CKD stages did not modify the IL6-CV risk relationship (P for interaction=0.33). Further analyses investigating IL-6 as a continuous variable confirmed this biomarker as a strong and independent risk factor for study outcomes [History of CVD, bivariate analysis OR (1 pg/mL): 1.16, 95% CI: 1.10-1.26, P<0.001; multivariate analysis OR: 1.10, 95% CI: 1.02-1.20, P=0.02; Incident CV events, bivariate analysis HR (1 pg/mL): 1.14, 95%CI: 1.07-1.22, P<0.001; multivariate analysis HR: 1.12, 95% CI: 1.03-1.22, P=0.007].

To account for the potential effect of competing risks due to death on the IL6-CV outcomes relationship, an additional adjusted analysis by considering a combined end-point death/CV events was performed. This multivariate analysis showed that the hazard ratio of the combined end point was about 1.5 times higher (HR:1.54, 95% CI:1.05-2.26, P=0.03) in patients with IL-6 levels above the median as compared with the remaining ones.

***Analyses based on the -174 G/C polymorphism***

In CKD patients, the genotype distribution of -174 G/C polymorphism [GG, n=333 (44%); GC, n=370 (49%); CC n=52 (7%)] significantly deviated from Hardy-Weinberg Equilibrium ($\chi^2$=14.3, P=0.001) while this was not true in healthy subjects [GG, n=277 (60%); GC, n=170 (37%); CC n=16 (3%)] ($\chi^2$=2.70, P=0.10). Of note, the frequency of the C risk allele was significantly higher (P<0.02) in CKD patients (31%, 95% CI: 28-34%) than in healthy study participants (22%, 95% CI: 18-26%).

CKD patients with CC genotype had higher levels of IL-6 (median:2.9 pg/ml, inter-quartile range:1.7-6.9 pg/ml) than those with GC (2.4 pg/ml, 1.6-3.8 pg/ml) and GG (2.5 pg/ml, 1.7-4.0 pg/ml) (P=0.04) (Figure 2, left panel) and this was also true for CRP (CC, median: 3.2 mg/L, IQR: 1.2-10.4 mg/L; GC, 2.2 mg/L, 1.0-5.6 mg/L; GG, 2.4 mg/L, 1.0-5.1 mg/L) (P=0.03) (Figure 2, right panel).

These findings show that the relationship between -174 G/C polymorphism and inflammatory biomarkers is well described by a recessive model of inheritance (CC

genotype versus GC/GG genotypes). For this reason, further data analysis was carried out by using the recessive model.

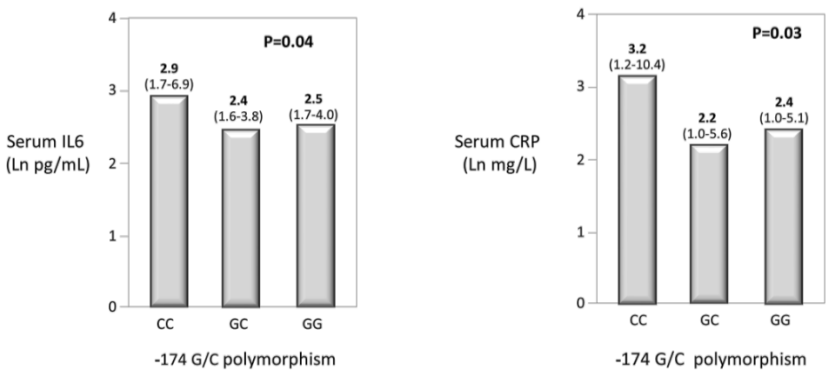


**Figure 2**. Serum levels of IL-6 (left panel) and C reactive protein (right panel) according to -174 G/C polymorphism in IL-6 gene. Data are expressed as medians. The inter-quartile ranges are indicated in parentheses. The comparison among groups was made by ANOVA

As shown in Table 4 and as expected from Mendelian randomization, CC patients versus GC/GG patients did not differ as for demographic and clinical characteristics except for a slight excess of males (75% versus 59%, P=0.02) in CC patients and slightly lower cholesterol levels (173±43 mg/dl versus 188±45 mg/dL, P=0.02) in patients with the same genotype.

The prevalence of the history of CVD was higher in CC patients (44%) than in GC/GG patients (28%) (P=0.01). The odds ratio for this outcome was more than twice higher in patients harboring the CC risk genotype (odds ratio: 2.15, 95% CI:1.15-4.00; P=0.02) than in those with other genotypes in a model adjusting for age, gender and cholesterol, i.e. the covariates which differed (P<0.10) between patients with CC and GC/GG genotypes (see Table 4). A bootstrapping validation model confirmed these results (odds ratio: 2.14, 95% CI:1.18-3.89; P=0.01).

**Table 4**. Main demographic and clinical characteristics of the study population according to -174 G/C polymorphism

| | **CC genotype** (n= 52) | **GC/GG genotype** (n= 703) | P |
|---|---|---|---|
| Age (years) | 59±13 | 62±10 | 0.09 |
| Males (%) | 39 (75) | 414 (59) | 0.02 |
| Diabetes (%) | 21 (40) | 242 (34) | 0.38 |
| Current Smokers (%) | 9 (17) | 89 (13) | 0.34 |
| CV comorbidities (%) | 23 (44) | 198 (28) | 0.01 |
| BMI (kg/m2) | 27.9±3.8 | 28.2±4.7 | 0.54 |
| Systolic BP (mmHg) | 132±16 | 134±18 | 0.41 |
| Diastolic BP (mmHg) | 76±10 | 78±10 | 0.15 |
| On anti-hypertensive treatment (%) | 44 (85) | 647 (92) | 0.69 |
| Glucose (mg/dL) | 98 (87-127) | 99 (88-120) | 0.65 |
| Total cholesterol (mg/dL) | 173±43 | 188±45 | 0.02 |
| Haemoglobin (g/dL) | 12±2.0 | 13±1.8 | 0.29 |
| Albumin (g/dL) | 4.1±0.4 | 4.2±0.5 | 0.53 |
| CRP (mg/L) | 3.2 (1.2-10) | 2.3 (1.0-5.4) | 0.06 |
| Phosphate (mg/dL) | 3.71±0.68 | 3.72±0.78 | 0.92 |
| eGFR (ml/min/1.73m2) | 34±12 | 36±13 | 0.32 |
| Urinary protein (g/24h) | 0.7 (0.2-1.6) | 0.6 (0.2-1.5) | 0.53 |

Data are expressed as mean ±standard deviation, median and inter-quartile range and as percent frequency, as appropriate.
Abbreviation: BMI: body mass index, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate

In the cohort study, the incidence rate of mortality did not differ between patients with and without the CC risk genotype (P=0.27) indicating that the competing risk of death on the -174 G/C polymorphism-CV events link could be excluded. In close parallelism with the strong association between serum IL-6 levels and CV events, the incidence rate of CV outcomes in patients with CC genotype was by the 87% higher (HR:1.87, 95% CI:1.02-3.44; P=0.04) than in those with the GC or GG genotypes (Figure 1, right panel) and this was also true in a bootstrapping validation model (HR:1.87, 95% CI:1.01-3.51; P=0.05).

**DISCUSSION**

In this study high serum IL-6 levels are associated with history of CVD and predict the risk for incident CV events in stage 2-5 CKD patients. Furthermore, the functional polymorphism -174 G/C in the promoter of IL-6 gene is associated with history of CVD and predicts the risk for future CV events in this population. These results are compatible with the hypothesis that this inflammatory cytokine is causally implicated in the high CV risk in CKD.

Atherosclerosis is an inflammatory disease (1). Chronic inflammation is pervasive at all CKD stages, particularly in stage 5D CKD patients (13, 14). IL-6 is considered as an orchestrator of the inflammatory response (37) and a key player in atherosclerosis in humans (38). The IL-6 effect on the cardiovascular system might be mediated via downstream acute-phase proteins (39) or by IL-6 per sé. IL-6 stimulates endothelial activation, vascular smooth muscle cell proliferation (40) and leukocyte recruitment (41), thereby contributing to the process of atherosclerotic plaque growth (42) and instability (43). IL-6 mRNA is overexpressed in atheromatous arteries and IL-6 expression co-localizes with macrophages in areas of plaque rupture (43). In the apoE-deficient mice, systemic administration of IL-6 accelerates atherosclerosis (10). Elevated levels of IL-6 are predictive of future CV events in healthy men (15) and women (44) and are markers of poor prognosis in patients with chronic angina (45) and acute coronary syndrome (46). IL-6 levels are markedly elevated in CKD, a phenomenon only in part explained by reduced renal clearance of this cytokine. Furthermore, the observation of a parallel increase in IL-6 and CRP in our study is in keeping with the notion that IL-6 drives the synthesis of CRP. High IL-6 has been solidly associated with mortality in stage 5 CKD patients maintained on chronic dialysis (16-20). To the best of our knowledge, there is only one study which tested the relationship between IL-6 and CV mortality in CKD (21). This seminal study was carried out in a small cohort of 125 patients, was based on a quite limited number of CV events (n=22) and included also dialysis patients (34% of the whole cohort) (21). Our study, based on a large CKD cohort composed exclusively by pre-dialysis patients and

including a large number of CV events, confirmed pilot data by Barreto et al. (21) and showed that high IL-6 is coherently associated both with history of CVD as well as with incident CV events.

Being observational in nature, findings in studies discussed above, including our analysis based on circulating IL-6, remain hypothesis generating and as such leave unresolved the critical question whether this cytokine is causally implicated in CV complications in pre-dialysis CKD patients. This question can only be resolved by a full-fledged clinical trial.

The Mendelian randomization approach is a useful step in the pathway to discovery in clinical research. Since genetic polymorphisms are distributed randomly at gamete formation and since genotypes precede phenotypes and do not change over time, comparing individuals harboring a given risk allele for the expression of a corresponding risk factor with those without the risk allele in question may allow unbiased assessment of the link between the attendant risk factor and relevant clinical outcomes. In this perspective, we used the functional polymorphism -174 G/C in the promoter of IL-6 gene as a marker to further investigate the link between IL-6 and CV events in CKD patients. The -174 G/C polymorphism is a common variant that regulates the serum concentration of IL-6 (22-28). In keeping with previous studies in patients with cardiovascular disease (28), coronary artery bypass grafting surgery (23, 25), carotid atherosclerosis (24), abdominal aortic aneurysm (22) and patients in dialysis (26), we found that CKD patients with CC genotype had higher circulating levels of IL-6 and CRP than those harboring GC or GG genotypes specifically legitimating the use of this genetic marker as an unbiased means for assessing the causal nature of the link between the gene product (IL-6) of this polymorphism and CV complications in CKD. Interestingly, this analysis showed that this polymorphism is independently associated both with the history of CVD as well as with incident CV events. Such associations, which went along with the previously described relationships of serum IL-6 with the same outcomes, provide further strength to the hypothesis that IL-6 is a direct player in atherosclerotic complications in CKD.

Furthermore, the observation that the distribution of genotypes frequency of the -174 G/C polymorphism in CKD patients was not in Hardy-Weinberg equilibrium and that the frequency of the C allele was significantly higher in CKD patients as compared with general population of the same geographical area, offers additional circumstantial evidence that IL-6 is a causal risk factor for CV events in this population (47).

Mendelian Randomization studies support causal interpretations but do not constitute definitive proof for causality, which demands specific experimental evidence (i.e. a formal randomized clinical trial). In this respect, meta-analytic data from two genetic consortia exploring the effect of a polymorphism in the interleukin-6 receptor (IL6R) on the risk of coronary artery disease, showed that the allele which attenuated IL-6 signaling was significantly associated with reduced risk of coronary heart disease (48). Notably, the relevance of this genetic association for the control of inflammation is showed by a meta-analysis of clinical trials testing a monoclonal antibody against the IL6R (Tocilizumab) in rheumatoid arthritis and documenting that lowering serum levels of IL-6 is an effective strategy to induce the remission of this chronic inflammatory disease (49).

Some limitations should be acknowledged. First, even though our cohort study registered a sizeable number of cardiovascular events, the number of deaths (n=42) was limited, preventing adequately adjusted analyses focusing on this major outcome. However, the analysis of the primary outcome in this study, incident CV events, which was robustly based on 117 events, showed parallel links between serum IL-6 and the genetic marker of this cytokine with the same events. Second, although Mendelian randomization is a powerful approach for inferring causality in observational studies, there are potential limitations to its application. Genetic variants can be indicators of environmental exposures on condition that there is no genetic admixture, pleiotropy or linkage disequilibrium. However, it is reasonable to believe that in our study all these assumptions are fulfilled. Our population is genetically homogeneous (29) and the -174G/C polymorphism is a functional variant directly responsible for serum levels of IL-6 (22-28). Furthermore, pleiotropy seems highly unlikely because of the location

of -174 G/C polymorphism in the promoter region of the gene. Third, despite we demonstrated that our findings had high internal validity by bootstrap modeling, replication of the results in a second cohort is a required proof for the external generalizability of findings in observational studies. In this respect, our observational study in a large Southern European cohort is a confirmation of a small central European cohort (21). Furthermore, our study is the first applying a genetic marker of IL-6 to express the nature (causal versus not causal) of the IL6-CV events link in CKD patients.

In conclusion, high serum IL-6 is associated both with history of CVD as well as with future CV events in CKD patients and these associations are fully confirmed by the application of a functional polymorphism in the IL-6 gene. Overall, this study is compatible with the hypothesis that the IL-6 plays a causal role in the high CV risk of CKD patients.

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## CHAPTER 5

# The fat-mass and obesity-associated gene (FTO) predicts mortality in chronic kidney disease of various severity

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**ABSTRACT**

**Background:** Polymorphisms in the FTO (fat-mass and obesity-associated) gene have been associated with body mass index, cancer, type 2 diabetes and hypertension.

**Methods:** We investigated the relationship between 17 tag single nucleotide polymorphisms (SNPs) and all-cause mortality in 3 cohorts of dialysis patients (CREED-1, North Apulian and CREED-2 cohorts; n=783) and in 1 cohort of stage 2-5 CKD patients (n= 757).

**Results:** We first explored the association between the 17 tag SNPs and all-cause mortality in the CREED-1 cohort and found that patients with the A allele of the FTO rs708259 polymorphism had an elevated risk of mortality (HR: 1.52, 95% CI: 1.11-2.08; P=0.008). Similarly, the A allele was associated with an increased risk of death also in the other two dialysis cohorts (North Apulian cohort, risk: + 23%; CREED-2 cohort, risk: +21%). The elevated risk portended by this allele was even higher in the stage 2-5 CKD cohort (+97%). However, the risk of mortality associated with the A allele in the 3 confirmatory cohorts failed to achieve formal statistical significance. In a meta-analysis including the 4 cohorts (n=1540; total deaths, n=381), individuals with the A allele had a 42% excess risk of death (HR:1.42, 95% CI: 1.14-1.76, P=0.002).

**Conclusion:** The A allele of the FTO rs708259 polymorphism is an independent predictor of all-cause mortality in patients with CKD of various severity. These data support our hypothesis that the FTO gene may be a relevant genetic risk factors for mortality in this population.

**INTRODUCTION**

Obesity is an expanding epidemic and a public health priority worldwide. The causes of this epidemic are mainly environmental because obesity clusters with social contacts (1). Genetic background contributes substantially to the risk of obesity and it is estimated that genetic factors explain about a half of the variation in adipose tissue mass (2). Over the last two decades, a large series of genes associated with human obesity have been identified and for most of these genes the association with obesity has been replicated (3). Among these genes, the FTO (fat-mass and obesity-associated gene) appears of particular interest because, beyond obesity, polymorphisms in this gene have been associated with mortality (4), cancer (5, 6, 7), diabetes (8, 9, 10) and hypertension (11).

Chronic kidney disease (CKD) is an emerging public health priority in economically developed and developing countries (12). The relationship between high body mass index (BMI) and survival in pre-dialysis (13) and dialysis (14) CKD patients seems to be complex. At variance with the general population, where excess adiposity is directly and linearly associated with the risk of death, the same association in CKD and in dialysis patients is either U shaped or inverse, suggesting that a high body mass may be protective in this population. Whether genetic variability in the FTO gene associates with elevated mortality in patients with chronic kidney disease has not been investigated. The issue is of relevance because diabetes and hypertension, two risk factors which have been associated to the FTO gene, rank as major risk factors for CKD while dialysis patients have an exceedingly high risk of incident cancers and diabetic patients on dialysis have a short survival. With this background in mind, we have therefore designed a genetic association study testing whether the variability of the FTO gene may contribute to explain mortality in CKD patients of various severity.

## SUBJECTS AND METHODS

### *Patients*

In the present study we included three independent cohorts of dialysis patients (the CREED-1 cohort, the North Apulian cohort and the CREED-2 cohort) with a total number of 783 patients and one cohort of stage 2-5 CKD patients (see **Table 1)**.

**Table 1.** Main characteristics of dialysis patients (CREED, North Apulian and CREED 2 cohorts) and of CKD patients

| | **CREED-1 cohort** | **North Apulian cohort** | **CREED-2 cohort** | **CKD cohort** |
|---|---|---|---|---|
| n | 265 | 220 | 298 | 757 |
| Age (years) | 61±15 | 58±16 | 61±15 | 62±11 |
| Male gender (%) | 56% | 53% | 63% | 60% |
| Diabetes (%) | 12% | 12% | 24% | 35% |
| BMI ($kg/m^2$) | 25±4 | 23±4 | 26±5 | 28±5 |
| Systolic BP (mmHg) | 140±24 | 142±19 | 136±22 | 134±18 |
| Diastolic BP (mmHg) | 77±13 | 82±8 | 74±12 | 78±11 |
| Enrolment period | Jan 1997–Feb 1998 | Nov 1999-May 1999 | May 2009-Oct 2010 | Oct 2005-Nov 2007 |

Data are expressed as mean ± SD or as percentage, as appropriate.

CREED-1 (15) included an incident-prevalent cohort of 265 dialysis patients (age 61±15 years; 56% Males) treated in the Urban Areas of Reggio Calabria (Calabria Region) and Catania (Sicily Region). These patients (all Caucasian) have been on regular dialysis treatment for at least 6 months, with left ventricular ejection fraction $\geq$ 35% and without circulatory congestion, major infections (fever, infected vascular access or peritonitis or exit site infection) or inter-current illnesses requiring hospitalization. Two hundred and fourteen haemodialysis patients were being treated thrice weekly with standard bicarbonate dialysis (Na 138 mmol/L, HCO3 5 mmol/L, K 1.5 mmol/L, Ca 1.25 mmol/L, Mg 0.75 mmol/L) either with Cuprophan or semi-synthetic membranes. The remaining 51 patients were on chronic ambulatory peritoneal dialysis (CAPD). One

hundred and five patients were habitual smokers and 109 patients were treated with anti-hypertensive drugs. The incident/prevalent North Apulian cohort included 220 dialysis patients (age 58±16 years; 53% Males) (16) enrolled in the health district of Foggia (Puglia region). Two-hundred and ten patients were treated thrice weekly with standard bicarbonate dialysis (Na 138 mmol/L, $HCO_3$ 5 mmol/L, K 1.5 mmol/L, Ca 1.25 mmol/L, Mg 0.75 mmol/L) by Cuprophan or semi-synthetic membranes and 10 patients were on CAPD. Fifty-three patients were habitual smokers and 175 were on anti-hypertensive treatment. The CREED-2 cohort included 298 hemodialysis patients non overlapping with the CREED-1 cohort. All patients in CREED-2 were of Caucasian descent and were being treated in 11 dialysis units in two regions of Southern Italy (Calabria and Sicily) and showed the same characteristics of CREED-1 and North Apulian cohort patients (see **Table 1**). All patients had been on regular hemodialysis with standard bicarbonate dialysis for a median time of 42 months (inter-quartile range: 20-81 months) and were being treated with non-cellulosic membrane filters of various type. One hundred and fifty-nine patients were habitual smokers and 174 patients were treated with anti-hypertensive drugs. The CKD cohort included 757 consecutive patients with stage 2-5 CKD of various aetiology. These patients were recruited from 22 Nephrology units in Southern Italy. All patients were in stable clinical condition and none had intercurrent infections or acute inflammatory processes. The large majority of patients (97%) were being treated with anti-hypertensive drugs (51% were on mono/double therapy with ACE-inhibitors, calcium antagonists, angiotensin II receptor antagonists, diuretics, alfa and beta blockers, and clonidine and the remaining 49% were assuming three or more various combinations of these drugs). Inclusion criteria were: non acute or rapidly evolving renal diseases; age ranging from 18 to 75 years; non-transplanted; non-pregnant, not affected by cancer or diseases in the terminal phase. The main characteristics of these four cohorts were given in **Table 1**.

***Follow-up study***

After the initial assessment, dialysis patients were followed up for a median time of 44 months (range: 0.20-154 months) in the CREED-1 study, for 66 months (range: 1-90 months) in the North Apulian cohort and for 26 months (range: 0.5-33 months) in the CREED-2 cohort. In the CKD cohort, patients were followed up for a median time of 33 months (range: 1-49 months). In all cohorts, during the observation period, all-cause mortality was accurately recorded and classified by trained outcome assessors.

***Haplotype structure and SNP selection***

The haploblock structure of the FTO gene for the Central European population was defined using Haploview (http://www.broadinstitute.org/haploview/haploview) (version 3.0 release R2, accessed June 2009; Whitehead Institute for Biomedical Research, USA). Using a minor allele frequency $\geq$ 5%, a pair-wise approach and setting an $r^2 \geq 0.80$, 16 single nucleotide polymorphisms (SNPs) (rs10163276; rs10521304; rs11075999; rs1125338; rs13334214; rs1078013; rs12935710; rs4784353; rs708259; rs7204916; rs8047395; rs860713; rs9924877; rs8044769; rs9926180; rs8050136), which were not in linkage disequilibrium between them, were sufficient to tag the haploblocks considered capturing most of the variability in the region. In addition, we determined the rs9939609 SNP which was in linkage disequilibrium with the rs8050136. This polymorphism, which maps in intron 1, has been repeatedly associated with fat mass in overweight and obese patients in previous large studies (8, 17, 18, 19).

***Genotyping of the selected SNPs***

Allelic discrimination of the selected 17 SNPs was performed using TaqMan SNP Genotyping Assays provided by Applied Biosystems on a 7900HT Fast Real-Time PCR platform and its accompanying Sequence Detection System (SDS) Software version 2.4 (Applied Biosystems, Foster City, CA, USA). Genomic DNA was extracted from peripheral blood leukocytes by salting-out technique (20). The reaction system contained 20 ng of genomic DNA, 12.5 μl of 2 X TaqMan Universal PCR Master Mix No AmpErase UNG, 1,25 μl of 40 X Assay Mix (including unlabeled PCR primers, FAM and

VIC dye-labeled TaqMan MGB probes) and $H_2O$ for a total volume of 25 μl. A random 10% of samples were independently repeated to confirm genotyping results. The genotype results for these samples were completely consistent.

***Laboratory measurements***

In the whole study population blood sampling was performed after an overnight fast always during a mid-week non-dialysis day for haemodialysis patients and at empty abdomen for CAPD patients. Blood was drawn and put into tubes containing EDTA, and plasma supernatants were stored at - 80°C until batch analyses. All analyses were done blinded to clinical information. Serum cholesterol, albumin and haemoglobin measurements were made using standard methods in the routine clinical laboratory.

***Statistical analysis***

Data were expressed as mean ± SD, median and inter-quartile range (IQR) or as percent frequency and comparisons between groups were made by independent T-Test, Mann-Whitney U test or Chi Square Test, as appropriate.

The relationship between FTO rs708259 polymorphism and all-cause mortality was investigated by Cox regression analysis in which the centre effect was accounted by a stratified analysis. As potential confounders we considered: Framingham risk factors (age, gender, smoking, diabetes, cholesterol and arterial pressure), anti-hypertensive treatment and factors peculiar to kidney failure (dialysis vintage, haemoglobin and albumin). A variable was considered as a confounder when it was related to both the exposure under investigation (the FTO rs708259 polymorphism) and the study outcome (all-cause mortality); was not an effect of the exposure and was not in the causal pathway between the exposure and outcome (21). For accounting for multiple testing, a Monte Carlo permutation analysis (10000 permutations) was done (22). This analysis provides an empirical P value (permuted P value) for the link between the FTO rs708259 polymorphism and all-cause mortality. The effect of the FTO rs708259 polymorphism on the risk of mortality was investigated separately in the four study cohorts as well as by a meta-analysis of these cohorts. The heterogeneity of the hazard ratios for death associated to the FTO rs708259 polymorphism among the four cohorts

was analysed by $I^2$ and Q-value (23). Data were expressed as hazard ratio (HR) and 95% confidence interval (CI) and P value. All calculations were made by using two standard statistical packages (SPSS for Windows – 9.01- Chicago, Illinois, USA and Comprehensive Meta Analysis – Version 2.2.064, BioStat, Englewood, NJ, USA).

**RESULTS**

We first investigated in the CREED-1 cohort study 17 SNPs (rs10163276; rs10521304; rs11075999; rs1125338; rs13334214; rs1078013; rs12935710; rs4784353; rs708259; rs7204916; rs8047395; rs860713; rs9924877; rs8044769; rs9926180; rs8050136; rs9939609) capturing most of the variability of the FTO gene. All these SNPs were in Hardy-Weinberg Equilibrium (P ranging from 0.09 to 0.98). Among these tag SNPs, the FTO rs708259 polymorphism was the only one to show an association with all-cause mortality (P=0.008) (see below) in this cohort of dialysis patients. Then, we extended the analysis to other two dialysis cohorts (North Apulian and CREED-2 cohorts) and tested the relationship of the same polymorphism with mortality in a fourth cohort of stage 2-5 CKD patients.

***FTO rs708259 polymorphism***

The FTO rs708259 polymorphism, either in CREED-1 study [GG, n=89 (33.0%); AG, n=129 (49.0%); AA, n=47 (18.0%), $\chi2$=0.001, P=0.98] or in North Apulian [GG, n=43 (19.0%); AG, n=96 (44.0%); AA, n=81 (37.0%), $\chi2$=2.22, P=0.14] and CREED-2 cohort [GG, n=94 (31%); AG, n=148 (50%); AA, n=56 (19%), $\chi2$=0.03, P=0.87] did not deviate from the Hardy-Weinberg Equilibrium. Similarly, in the CKD cohort (n=759), the genotypic distribution of the FTO rs708259 polymorphism was in Hardy-Weinberg Equilibrium [GG, n=257 (34.0%); AG, n=379 (50.0%); AA, n=121 (16.0%), $\chi2$=0.91, P=0.34].

In **Table 2** the main demographic and clinical characteristics of patients of the three combined dialysis cohorts (n=783) are described according to their genotypes.

Patients with AA or AG genotypes did not differ from those homozygotes for the G allele. The same analysis carried out separately in the three dialysis cohorts provided similar results (data not shown).

**Table 2.** Main demographic, somatometric and clinical characteristics of the dialysis population (CREED-1, North Apulian and CREED-2 cohorts)

| | **GG (rs708259) (n=226)** | **AG/AA (rs708259) (n=557)** | **P** |
|---|---|---|---|
| Age (years) | 60±15 | 60±16 | 0.49 |
| Male sex n. (%) | 128 (57) | 325 (58) | 0.66 |
| Smokers n. (%) | 93 (41) | 224 (40) | 0.82 |
| Diabetics n. (%) | 45 (20) | 96 (17) | 0.35 |
| Dialysis vintage (months) | 43 (18-99) | 44 (20-99) | 0.91 |
| *BMI (kg/m$^2$) | 24.9±4.5 | 25.2±5.0 | 0.42 |
| Systolic pressure (mmHg) | 140±22 | 139±22 | 0.34 |
| Diastolic pressure (mmHg) | 77±12 | 77±12 | 0.96 |
| On anti-hypertensive treatment n. (%) | 127 (56) | 331 (59) | 0.42 |
| Cholesterol (mg/dL) | 181±53 | 180±52 | 0.75 |
| Haemoglobin (g/L) | 11.3±1.7 | 11±1.6 | 0.11 |
| Albumin (g/L) | 3.95±0.48 | 3.87±0.51 | 0.06 |

Data are expressed as mean± SD. median and inter-quartile range or as percent frequency. as appropriate. Comparisons between groups were made by independent T-test, Mann Witney U test or Chi-Square Test, as appropriate.

**Symbol:**

* Available in 676 patients.

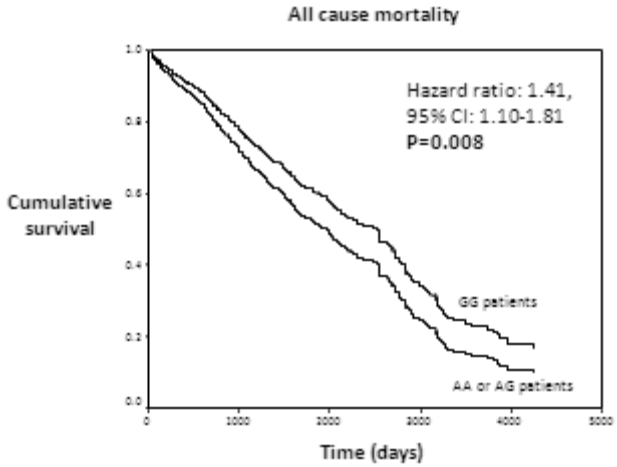
***FTO rs708259 polymorphism and survival in dialysis patients***

During the follow-up period (median: 44 months, range: 0.2-154 months) of the whole study population of dialysis patients (n=783), 339 patients died, 172 of them (51%) of cardiovascular causes. On univariate Cox regression analysis, the excess risk of death was 41% higher (hazard ratio: 1.41, 95% CI: 1.10-1.81, P=0.008; permuted P value=0.006) in patients with AA or AG genotypes than in those with GG genotype (**Figure 1**), and the excess risk in the three combined cohorts did not differ from that observed in the three cohorts considered separately (CREED-1 cohort, excess risk: +

52%; North Apulian cohort, excess risk: + 23%; CREED-2 cohort, excess risk: +21%) (**Figure 2**).

Adjustment for albumin (which was the only variable that tended to be different among genotypes) (see **Table 2**) had no material effect on the strength of the association between the FTO rs708259 polymorphism and the risk of mortality (HR:1.41, 95% CI: 1.10-1.82, P=0.007, permuted P value=0.005).

**Figure 1.** Kaplan-Meier survival curves of all-cause mortality in the whole dialysis population (CREED-1, North Apulian and CREED-2 cohorts; n=783) according to the genotypes of the FTO rs708259 polymorphism. Data are expressed as Hazard Ratio, 95% confidence intervals and P value.

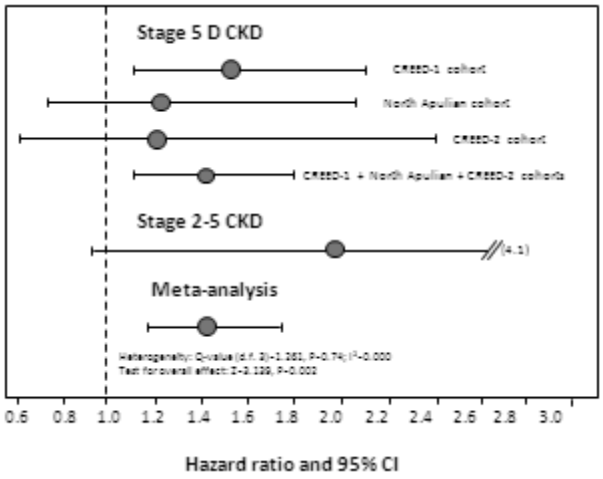



***FTO rs708259 polymorphism and survival in stage 2-5 CKD patients***

During the follow-up period (33 months, range 1-49 months), 42 CKD patients died, 31 (74%) of them of CV causes. On univariate Cox regression analysis, CKD patients having AA or AG genotypes of the FTO rs708259 polymorphism had an excess risk of death almost doubled (+97%) as compared to patients with GG genotype and this

excess risk did not differ (P=0.32) from that observed in dialysis patients. However, probably due to the small number of death cases in this cohort of pre-dialysis patients, such a risk excess failed to achieve formal statistical significance (HR:1.97, 95%CI: 0.94-4.11; P=0.07) in this cohort (**Figure 2**).

**Figure 2.** Hazard ratios (and 95% confidence intervals) of the FTO rs708259 polymorphism for all-cause mortality in the study cohorts. At the bottom of the figure, a pooled analysis of all cohorts based on a meta-analytic approach is reported




***Meta-analysis of the four study cohorts***

There was no effect heterogeneity (P=0.74) of FTO rs708259 polymorphism on the risk of mortality in patients with kidney failure and in those with stages 2-5 CKD and for this reason fixed- and random-effects models provided identical results. In a meta-analysis of the four cohorts (total number of patients, n=1540; total deaths, n=381), AA or AG individuals had a 42% excess risk of mortality as compared to those homozygotes for the G allele (HR: 1.42, 95% CI: 1.14-1.76, P=0.002) (**Figure 2**) further

confirming that the A allele of the FTO rs708259 polymorphism associates with reduced survival in CKD patients.

**DISCUSSION**

This study shows an association between the A allele of the FTO rs708259 polymorphism and all-cause mortality in pre-dialysis and dialysis patients.

***FTO gene and obesity***

The fat-mass and obesity-associated (FTO) gene has been repeatedly associated with various obesity traits (8, 17, 18, 19, 24), insulin resistance and type 2 diabetes (8, 9, 10). This gene codes for an enzyme that oxidatively demethylates single-stranded DNA (25) and, by this mechanism, it may modulate relevant epigenetic modifications of other fundamental genes regulating various biological processes. A common sequence variant in the first intron of this gene, the rs9939609, predisposes to type 2 diabetes through an effect on body mass index (BMI) (8) and an association between this polymorphism and the BMI has been replicated in 13 external cohorts including 38,759 European subjects (8). Furthermore, other FTO polymorphisms have been associated with severe obesity in individuals of French descent (18).

***FTO gene and renal disease***

Although studies performed so far have primarily focused on the association between the FTO gene and obesity, variants in this gene associate also with other major clinical conditions, including cancer (5, 6, 7), hypertension (11), Alzheimer's disease (26) and kidney failure (27). The FTO is one of the largest genes (more than 4 Mb) which have been implicated in human health. To study the association between genetic variants in FTO gene, we selected 17 tag SNPs that reflected the haploblock structure of the gene. Of these SNPs, none of those localized in intron 1 was associated with mortality. However, in the first cohort enrolled in this study (CREED-1 cohort), we identified a new polymorphism, the rs708259 on intron 8 of the FTO gene, which was strongly associated with death in dialysis patients. When we extended the analysis to two replication cohorts, the North Apulian and the CREED-2 cohort, which included dialysis

patients comparable for the main demographic and clinical characteristics to the first cohort, we observed again an excess risk of death (+23% and + 21%, respectively) in A-allele carriers. Importantly, such an association was also confirmed in a third independent cohort of stage 2-5 CKD patients. Although the associations between the rs708259 polymorphism and mortality did not attain formal statistical significance in the three confirmatory cohorts, a meta-analysis of the 4 cohorts showed that individuals harbouring the risk allele A of the FTO rs708259 polymorphism have a highly significant (p=0.002) 42% excess risk of death as compared to individuals without such an allele.

To the best our knowledge, this is the first study investigating the role of the FTO gene on mortality in pre-dialysis and dialysis patients. The FTO rs708259 polymorphism in intron 8 is not in linkage disequilibrium with any of the SNPs in the intron 1 of the gene. While the association between the FTO rs708259 polymorphism and mortality in pre-dialysis and dialysis patients in our data is statistically robust, the functional significance of this SNP is unknown. Because the FTO rs708259 polymorphism is unrelated to BMI and diabetes, it seems unlikely that the FTO gene affects survival through its impact on mechanisms regulating energy balance or glucose metabolism in this population. We speculate that this polymorphism may exert functional effects by modifying the bioavailability of the transcript and/or the protein product of FTO. Alternatively, this polymorphism may enhance the risk of mortality via DNA methylation. Functional studies in appropriate models are needed to mechanistically interpret the association between the FTO rs708259 polymorphism and mortality in the CKD population.

In conclusion, our findings generate the hypothesis that CKD patients of various severity with the A allele of the FTO SNP rs708259 polymorphism may have a higher death risk than those without such an allele. Functional studies will define the mechanism(s) whereby this polymorphism impacts upon survival in this population.

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## CHAPTER 6

# Resistin and all-cause and cardiovascular mortality: effect modification by adiponectin in end-stage kidney disease patients

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**ABSTRACT**

**Background:** Resistin is a major adipose tissue cytokine implicated in insulin resistance, inflammation and vascular damage. This cytokine is raised in patients with End-Stage Kidney Disease (ESKD) but the relationship between resistin and major clinical outcomes has not been investigated in this population.

*Methods:* We studied the mutual relationship between resistin and the two major adipokines (adiponectin and leptin) and the interaction between resistin and adiponectin (ADPN) and all-cause and cardiovascular (CV) mortality in a cohort of 231 hemodialysis patients followed up for 57±44 months.

**Results:** Plasma Resistin was substantially raised in ESKD patients as compared to healthy subjects (P<0.001). On univariate analysis, resistin was related inversely to ADPN (r=-0.14, P=0.04) and directly to C-Reactive Protein (r=0.15, P=0.03) but was largely independent of leptin (r=0.08, P=0.24) and HOMA-IR index (r=-0.04, P=0.51). During the follow-up, 165 patients died (96 for CV causes). On both univariate (all-cause mortality: P=0.004; CV mortality P<0.001) and multivariate (all-cause mortality: P=0.01; CV mortality P<0.001) Cox regression analyses the effect of resistin on study outcomes was closely dependent on ADPN levels. There was a consistent excess risk for all-cause (P=0.002) and CV mortality (P=0.003) by plasma resistin (20 ng/mL) in patients in the first ADPN tertile but no risk excess for these outcomes was apparent in patients in the third tertile.

**Conclusion:** This study indicates that resistin predicts death and fatal CV events depending on plasma ADPN levels. These findings underscore the importance of the interaction among adipokines for the prediction of adverse clinical outcomes in ESKD.

## INTRODUCTION

Over the past decade numerous clinical studies focusing on the relationship between two main adipokines, adiponectin (ADPN) and leptin, and major clinical events in end-stage kidney disease (ESKD) patients have been published (1-5). Resistin is a cysteine rich molecule of the "found in inflammatory zones" (FIZZ) proteins, which is synthesized in the adipose tissue (6, 7) and in macrophages (8, 9). High levels of this peptide go along with inflammation and insulin resistance both in experimental models (6, 10, 11) and in vivo in man (12-14). Interest on resistin in ESKD has been much limited and until now no study tested the relationship between this cytokine and major clinical outcomes, including all-cause and cardiovascular (CV) death, in this population. The issue is of relevance because high resistin associated with mortality and incident heart failure in patients with coronary heart disease (15) or with acute myocardial infarction (16). Furthermore, a most risky arrhythmia like atrial fibrillation was predicted by resistin levels in the Framingham Heart study population (17). Adipose tissue cytokines have mutual inter-relationships and interact with abdominal obesity in determining death and CV events (4). Furthermore, ADPN is a modifier of the relationship between leptin and fat mass in young individuals (18) and modulates the risk for type-2 diabetes by leptin in the general population (19).

With this background in mind, we identified the functional correlates of circulating resistin in ESKD patients and studied the relationship between resistin, death and incident CV events as well as the interaction between resistin and the adipokine which emerged as its strongest functional correlate, adiponectin, and all-cause and CV mortality.

## SUBJECTS AND METHODS

### *Protocol*

The protocol was in conformity to the ethical guidelines of our Institutions and informed consent was obtained from each participant. All blood samples for

laboratory tests were taken during a mid-week non-dialysis day, between 8 A.M. and 1 P.M.

***Patients and controls***

We studied an incident-prevalent cohort of 231 hemodialysis patients (127 M and 104 F) who had been on regular dialysis treatment (RDT) for at least 6 months. The enrolment criteria in this cohort were no history of congestive heart failure [defined as dyspnea in addition to two of the following conditions - raised jugular pressure, bibasilar crackles, pulmonary venous hypertension or interstitial oedema on chest X ray, requiring hospitalization or extra ultra-ultrafiltration], left ventricular ejection fraction >35% and no inter-current or terminal illnesses. Patients were treated thrice weekly with standard bicarbonate dialysis (Na 138 mMol/L, HCO3 35 mMol/L, K 1.5 mMol/L, Ca 1.25 mMol/L, Mg 0.75 mMol/L) either with cuprophan or semi-synthetic membranes (dialysis filters surface area: 1.1-1.7 $m^2$).

For comparison, we assessed resistin plasma concentration in 41 healthy volunteers.

***Laboratory measurements***

Blood sampling was performed after an overnight fast between 8.00 a.m. and 10.00 a.m. always during a mid-week non-dialysis. After 20-30 min of quiet resting in semi-recumbent position samples were taken into chilled EDTA vacutainers, placed immediately on ice, centrifuged within 30 min at -4°C and the plasma stored at -80°C before assay. Serum lipids, albumin, glucose, phosphate, and haemoglobin measurements were made using standard methods in the routine clinical laboratory. Plasma concentration of C-Reactive Protein (CRP), leptin and adiponectin were measured according to methods described elsewhere (1). Plasma levels of IL-6 and TNF $\alpha$ were measured by using commercially available kits (R&D Systems, Minneapolis, Minnesota). The intra-assay coefficient of variation for these molecules ranged from 2.6% to 4.7% and interassay coefficient of variation from 4.5 to 5.8%. Plasma resistin was measured by ELISA (Bio Vendor Laboratory Medicine Inc., Brno Czech Republic). The intra-assay coefficient of variation was 4.3% and the inter-assay coefficient of variation was 6.8%. The average plasma resistin in 41 healthy volunteers

was 10.8 ± 6.2 ng/mL with a normal range spanning from 1.2 ng/mL to 29.9 ng/mL. Serum insulin levels were measured by using a commercially available RIA kit (Sorin Saluggia, Vercelli, Italy). Insulin sensitivity was estimated by using the homeostatic model assessment (HOMA-IR) index [*i.e.*, plasma glucose level × (plasma insulin level/22.5)].

***Follow-up study***

After the initial assessment, patients were monitored for 57 ± 44 months (range: 0.2 to 155 months). During the follow-up period, fatal cardiovascular events (ECG documented myocardial infarctions, heart failure, ECG documented arrhythmia, strokes, peripheral vascular disease, or major arterial or venous thrombotic episodes) and death for other causes were accurately recorded. Each death was reviewed and assigned an underlying cause by a panel of five physicians. As part of the review process, all available medical information regarding each death was collected. This information always included study and hospitalization records. In the case of an out-of-hospital death, family members were interviewed by telephone, for better assessment of the circumstances surrounding the death.

***Statistical analysis***

Data were expressed as mean ± standard deviation (SD), median and inter-quartile range (IQR) or as percent frequency, as appropriate. Comparisons among groups were made by P for linear trend. The association between two continuous variables was assessed by the Pearson product moment correlation coefficients and between binary and continuous variables by the point-biserial correlation coefficient. Variables having a positively skewed distribution were log transformed ($lg_{10}$) before the correlation study. The effect modification of ADPN (i.e. the interaction analysis) (20) on the relationship between resistin and all-cause and cardiovascular mortality was investigated by univariate and multivariate Cox regression analyses. In multivariate models we included resistin, ADPN and their interaction term [resistin (20 ng/mL increase) * ADPN (tertiles)] as well as traditional risk factors (age, gender, smoking, systolic pressure, cholesterol, diabetes, previous cardiovascular events, BMI), factors

peculiar to ESRD (haemoglobin, albumin, phosphate, dialysis vintage) and CRP. By this strategy we built up models of adequate statistical power for all-cause mortality (at least 1 variable every 11 deaths). In these analyses ADPN was modelled in terms of tertiles because this functional form was the one that optimized data fitting (21).To account for over-fitting for cardiovascular mortality, a shrinkage analysis was performed (22). The proportionality assumption was tested by analysing the Schoenfeld residuals and no violation was found. The hazard ratios of a fixed increase in Resistin (20 ng/mL) across ADPN tertiles were calculated by the linear combination method. Data were expressed as hazard ratios, 95% CIs and P values. All calculations were done by standard statistical packages (SPSS for Windows Version 9.0.1, 11 Mar-1999, Chicago, Illinois, USA; STATA/SE 9.0 StataCorp LP, TX, USA).

## RESULTS

### *Patients*

The study population included 231 haemodialysis patients (age: 60±15 years; Males: 55%) on regular dialysis treatment for a median time of 41 months (inter-quartile 21-106 months) (see **Table 1**).

One hundred and fifteen patients (50%) had previous CV events, 35 were diabetic (15%) and 86 (37%) were habitual smokers (22±16 cigarettes/day). Eighty-four patients (36%) were on anti-hypertensive treatment (59 on mono-therapy with ACE inhibitors, AT-1 antagonists, Calcium channel blockers, alpha and beta-blockers and 25 on double or triple therapy with various combinations of these drugs). One hundred and twenty six patients (55%) were treated with erythropoietin. Average urea Kt/V was 1.22±0.27.

### *Correlates of plasma Resistin*

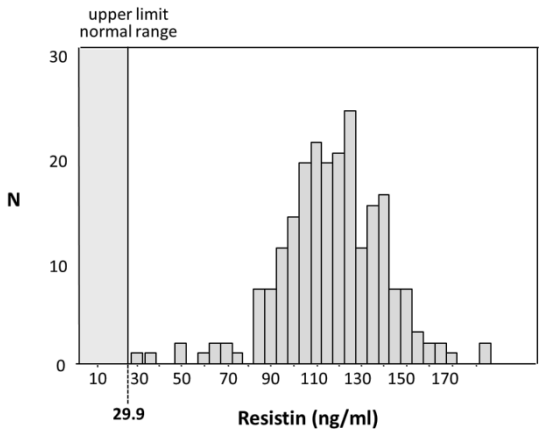
Plasma resistin in ESKD patients had a normal distribution with an average value of 117.2±23.3 ng/mL which was about 11 times higher than the average normal value (10.8±6.2 ng/mL) and exceeded the upper limit (29.9 ng/mL) of the normal range in all patients (**Figure 1**).

**Table 1.** Demographic, somatometric and clinical data of the study population

| | **Whole group** (n=231) |
|---|---|
| Age (years) | 60±15 |
| Male sex n. (%) | 127 (55) |
| BMI (kg/m$^2$) | 24.5±4.4 |
| Diabetics n. (%) | 35 (15.2) |
| Smokers n. (%) | 86 (37.2) |
| Patients with CV comorbidities n. (%) | 115 (50%) |
| Dialysis vintage | 41 (21-106) |
| Kt/V | 1.22±0.27 |
| On EPO treatment n. (%) | 126 (55%) |
| On-anti-hypertensive treatment n. (%) | 84 (36%) |
| Systolic BP (mmHg) | 139±25 |
| Diastolic BP (mmHg) | 76±13 |
| | |
| Total cholesterol (mg/dL) | 208.7±57.8 |
| Glucose (mmol/L) | 5.6±3.2 |
| Insulin (μU/mL) | 19.8(13.7-29.7) |
| HOMA-IR (μU/mL*mmol/L) | 4.0(2.7-6.8) |
| Haemoglobin (g/dL) | 10.7±1.9 |
| Albumin (g/dL) | 4.2±0.5 |
| Phosfate (mg/dL) | 4.5±1.1 |
| CRP (mg/L) | 7.4 (3.4-16.3) |
| IL-6 (pg/mL) | 5.0 (2.8-9.2) |
| TNF-α (pg/mL) | 5.6 (1.8-1.5) |
| Resistin (ng/mL) | 117.2±23.3 |
| Adiponectin (□g/mL) | 15.0±7.7 |
| Leptin (ng/mL) | 10.0 (4.8-30.7) |

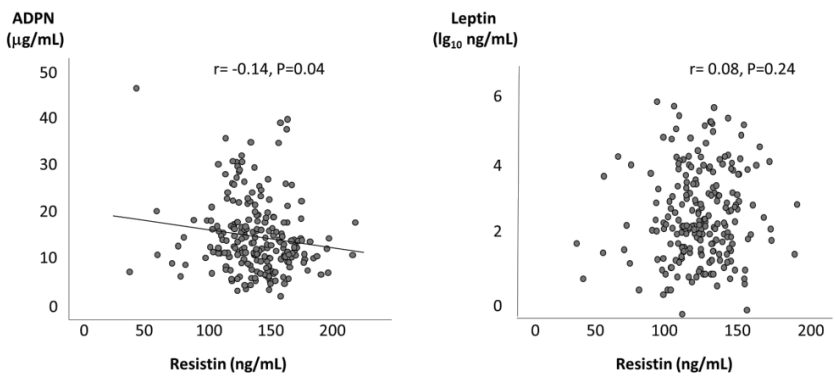
Data are expressed as mean± SD, median and inter-quartile range or as percent frequency, as appropriate.

**Figure 1.** Distribution of plasma resistin in the study population. All ESKD patients exceeded the upper limit (29.9 ng/mL) of the normal range (the grey area to the left of the graph).




Plasma resistin was weakly related to plasma ADPN (r=-0.14, P=0.04) (**Figure 2a**) but was largely unrelated with leptin (r=0.08, P=0.24) (**Figure 2b**) and with insulin (r=0.01, P=0.99) and HOMA-IR index (r=-0.04, P=0.51). Furthermore, resistin was weakly related with dialysis vintage (r=0.21, P=0.002), CRP (r= 0.15, P=0.03) and with age (r= -0.17, P=0.01).

**Figure 2.** Correlation between plasma resistin and adipokines.



***Survival analysis***

During the follow-up period, 165 patients died, 96 of them for CV causes. Resistin failed to significantly predict all-cause and CV mortality (P=NS). On univariate Cox regression analysis, patients in the lowest ADPN tertile had higher all-cause mortality rate (19 deaths/100 persons year) when compared to those in the second (11 deaths/100 persons year) and third ADPN tertile (17 deaths/100 persons year) (P for trend=0.02). The same analysis carried out for CV mortality provided similar results (ADPN, I tertile: 13 CV deaths/100 persons year; II tertile: 6 CV deaths/100 persons year; III tertile: 8 CV deaths/100 persons year)(P for trend =0.02). Formal interaction analysis showed that ADPN is a strong modifier of the relationship between resistin and study outcomes both on univariate (all-cause mortality: P for effect modification=0.004; CV mortality: P for effect modification<0.001) or on multivariate Cox regression analyses (**Table 2** and **Figure 3**).

Indeed, the risk excess for all cause and CV mortality portended by a fixed increase in resistin (20 ng/mL) was apparent and highly significant (all-cause mortality: P for effect modification=0.01; CV mortality: P for effect modification<0.001) in patients with low ADPN (first ADPN tertile all-cause death, HR: 1.32, 95% CI: 1.11-1.58; CV death, HR: 1.42, 95% CI: 1.12-1.79), slight and not significant in those in the second tertile (all-cause death, HR: 1.10, 95% CI: 0.95-1.27; CV death, HR: 1.04, 95% CI: 0.86-1.26) and absent (all-cause death, HR: 0.91, 95% CI: 0.73-1.14; CV death, HR: 0.77, 95% CI: 0.57-1.02) in patients in the third ADPN tertile (**Figure 3**).

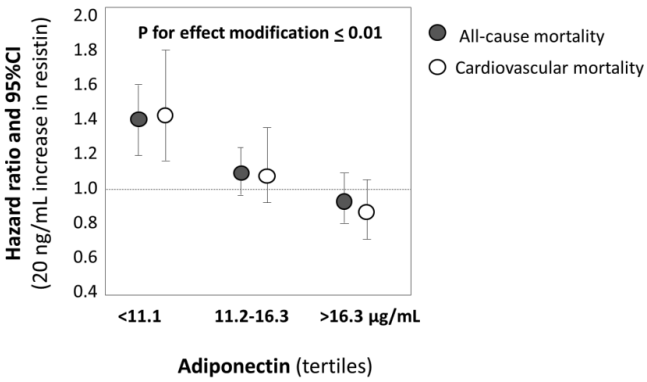
The effect modification by ADPN on the resistin-CV mortality link remained significant (P=0.006) also after Shrinkage correction accounting for over-fitting. No interaction was found between ADPN and resistin with CRP, IL-6 and TNF-α (all P=NS). Forcing plasma leptin, insulin and HOMA-IR into the multivariate models of all-cause and CV death did not affect the strength of the relationship between the resistin-ADPN interaction term and all-cause (P=0.01) and CV mortality (P<0.001).

**Table 2** Multiple Cox regression model of the adiponectin- resistin interaction for all-cause and cardiovascular mortality

| Variables (units of increase) | **All-cause mortality** | | **CV mortality** | |
|---|---|---|---|---|
| | Hazard ratio (95% CI) | *P value* | Hazard ratio (95% CI) | *P value* |
| Resistin (20 ng/mL) | | | | |
| ADPN tertiles, μg/mL (I$^{st}$ ≤11.1, II$^{nd}$ 11.1-16.3, III$^{rd}$>16.3) | **See Fig.3** (P for effect modification=0.01) | | **See Fig.3** (P for effect modification<0.001)* | |
| ADPN x Resistin | | | | |
| BMI (1 kg/m$^2$) | 1.02 (0.98 to 1.06) | 0.37 | 1.05 (0.99 to 1.10) | 0.09 |
| ***Framingham risk factors*** | | | | |
| Age (1 yr) | 1.04 (1.03 to 1.06) | <0.001 | 1.05 (1.02 to 1.07) | <0.001 |
| Male gender | 1.85 (1.20 to 2.86) | 0.005 | 1.79 (1.01 to 3.18) | 0.05 |
| Smoking | 1.16 (0.78 to 1.74) | 0.72 | 1.47 (0.88 to 2.43) | 0.14 |
| Systolic pressure (1 mmHg) | 0.99 (0.99 to 1.01) | 0.48 | 1.00 (0.99 to 1.01) | 0.40 |
| Cholesterol (1 mg/dL) | 1.01 (0.99 to 1.01) | 0.37 | 1.00 (1.00 to 1.01) | 0.10 |
| Diabetes | 2.34 (1.50 to 3.67) | <0.001 | 2.29 (1.30 to 4.03) | 0.004 |
| Previous cardiovascular events | 1.80 (1.27 to 2.56) | 0.001 | 2.20 (1.37 to 3.52) | 0.001 |
| ***Factors peculiar to ESRD*** | | | | |
| Haemoglobin (1 g/dL) | 1.09 (0.99 to1.20) | 0.09 | 1.19 (1.04-1.36) | 0.01 |
| Albumin (1 g/dL) | 0.72 (0.50 to 1.04) | 0.08 | 0.82 (0.5 to 1.36) | 0.45 |
| Phosphate (1 mg/dL) | 1.04 (0.92 to 1.19) | 0.50 | 1.05 (0.89 to 1.25) | 0.57 |
| Dialysis vintage (1 month) | 1.00 (0.99 to 1.01) | 0.80 | 1.00 (1.00 to 1.01) | 0.58 |
| ***Emerging risk factors*** | | | | |
| CRP (1 mg/L) | 1.01(1.00 to 1.01) | 0.03 | 1.00 (0.99 to 1.01) | 0.75 |

Data are expressed as hazard ratio, 95% CI and P value.
*P=0.006 after Shrinkage correction for over-fitting (see Methods-Statistical Analysis for more details).

**Figure 3.** Effect modification of plasma adiponectin (ADPN) (expressed in tertiles) on the link resistin-mortality. The risk excess for all-cause and CV mortality portended by a fixed increase in resistin (20 ng/mL) is highly significant in patients in the first ADPN tertile, slight and not significant in those in the second tertile and absent in patients in the third tertile. Data are expressed as hazard ratios, 95% CIs and P values.




**DISCUSSION**

Resistin is markedly raised in ESKD patients, is weakly related with inflammation, does not associate with insulin sensitivity and correlates inversely with ADPN. Furthermore, the association of resistin with all-cause death and fatal CV events is strongly dependent (effect modification) on concurrent ADPN levels, being apparent and highly significant only in patients with low ADPN. This finding implies that high ADPN acts as a factor protecting from the noxious health effects of increased resistin in this population.

Adipocytes are considered as the sole source of resistin in mice (6, 7) where this adipokine is implicated in insulin resistance and hyperglicemia (6, 7, 10). In man this molecule is expressed primarily in macrophages and is involved in inflammatory processes (8, 13). It is interesting to note that plasma resistin correlates more closely with inflammation than with insulin resistance in overweight subjects as well as in

patients with chronic kidney disease (23-25). Plasma resistin is markedly elevated in ESKD (26, 27) and it is still debated if this phenomenon is a mere consequence of accumulation secondary to reduced renal clearance and if it may also reflect chronic inflammation (27). We found that, resistin in ESKD patients was not only higher than in normal subjects but also significantly related with serum level of CRP while it did not correlate with insulin resistance as assessed by circulating levels of insulin and HOMA-IR index, a finding confirming observations in pre-dialysis CKD patients (26). Resistin exerts a pro-inflammatory effect on vascular endothelial cells via up-regulation of endothelin-1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) (28, 29), which are all critical mediators of the early steps of the atherosclerosis process (30). In diabetic and non-diabetic patients, plasma resistin levels associate with inflammatory markers as well as with coronary artery calcification (14). Of note, recent evidence points to resistin as a cogent predictor of incident coronary heart disease (CHD) and congestive heart failure (CHF) in the general population (31). High resistin is also strongly and independently associated with major CV events and all-cause mortality in type 2 diabetes patients (32). However, in patients with stable CHD, the association between resistin and mortality in an unadjusted analysis was largely due to confounding by traditional CV risk factors and CKD (15).

***Risk for all-cause and CV death associated with resistin in ESKD***

The association between resistin and major clinical outcomes like mortality and CV events was tested in two studies in patients with coronary heart disease (15) or fatal myocardial infarction (16) and in both studies high resistin signalled a high death risk or an increased incidence of heart failure. ESKD patients are a population with exceedingly high risk for death and CV events (33). Causal risk factors for such a high risk are still not well defined and therefore testing the hypothesis that resistin is implicated in this high risk is a relevant question with etiologic and prognostic implications. In this regard, our study is the very first investigating the relationship between resistin and major clinical outcomes in ESKD. We found that resistin

predicted death and fatal CV outcomes in individuals with low ADPN but not in those with normal or high ADPN. This association was statistically strong and quantitatively of potential clinical relevance. The effect modification by ADPN on the relationship between resistin and mortality and fatal CV events has biological plausibility because ADPN and resistin have opposite effects on endothelial function and on the atherosclerotic process, resistin being a noxious factor for the CV system and ADPN a protective one. Of note, in our study the effect modification by ADPN on the resistin-mortality and resistin-CV mortality relationships was fully independent of potential confounders, including traditional CV risk factors as well as risk factors peculiar to ESKD, including haemoglobin, albumin and CRP.

In conclusion, we found that in ESKD, ADPN is a modifier of the link between resistin and mortality. Indeed, the risk for all-cause and CV mortality portended by a fixed increase in plasma resistin is evident in patients with low levels of ADPN but absent in those with high levels of this adipokine. These data underscore the importance of interaction analysis among adipokines to fully capture the relevance of their associations with adverse clinical events in the ESKD population.

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## CHAPTER 7

# Oxidized LDL, Gamma-glutamyltransferase (GGT) and adverse outcomes in older adults

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**ABSTRACT**

**Background/Objectives:** Gamma-glutamyltransferase (GGT) is a biomarker of liver disease and oxidative stress which was associated with all-cause and cardiovascular (CV) mortality in the general population and in patients with high risk conditions. This study aims at assessing whether oxLDL modifies the relationship between GGT, all-cause and CV mortality in elderly individuals from the general population.

**Design:** Observational longitudinal study.

**Setting:** Population-based cohort of older individuals (>65 years) free of liver disease.

**Participants:** One thousand and thirty-eight individuals from the Invecchiare in Chianti (InCHIANTI) study.

**Measurements:** serum GGT level, oxidized low-density lipoprotein (oxLDL), CV comorbidities, all-cause and CV mortality.

**Results:** The median age of the study population (n=1038) was 74 years (inter-quartile range: 69-79), 152 individuals (15%) had past CV events. During a median follow-up of 9 years, 401 individuals died, 168 of them (42%) for CV causes. In adjusted analyses, GGT predicted all-cause mortality (HR for 20U/L increase in serum GGT:1.11, 95% CI:1.02-1.21, P=0.02) and CV mortality (HR:1.17, 95%CI:1.03-1.33; P=0.02). Furthermore, in an analysis for interaction circulating oxLDL amplified the effect of GGT on all-cause mortality (P=0.003).

**Conclusions:** Circulating oxLDL amplifies the effect of GGT on mortality in the elderly. The mechanism for this association remains unknown and requires further research, including studying the potential role of GGT in oxidative stress. These results are consistent with the hypothesis of a causal role of GGT in the CV morbidity and mortality in older individuals and indicate that oxLDL plays a crucial role in the interpretation of the link between GGT and the risk of adverse clinical events in this population.

## INTRODUCTION

Aging is characterized by a progressive decline of anatomic integrity and function across multiple tissues and organs. A number of mechanisms have been proposed to drive the aging process, including accumulation of damaged macromolecules due to oxidative stress (1).

Gamma-glutamyltransferase (GGT), a multifaceted biomarker impinging upon oxidative stress (2,3), emerged as a risk factor for all-cause and cardiovascular (CV) mortality in population-based studies independent of liver disease and alcohol intake (4, 5). GGT has been detected within atherosclerotic plaques of cerebral and coronary arteries where it co-localizes with oxidized low-density lipoprotein (oxLDL) (6, 7). In theory, such a co-localization may be key to the interpretation of oxidative stress damage in the arterial system.

In the sole study in an elderly cohort testing the relationship between GGT and mortality and CV events, this biomarker was a direct predictor of adverse clinical outcomes (8) which contrasts with age-stratified analyses in community-based studies where GGT predicted mortality in the young and middle age strata but not in the elderly (9-12). Herein, we tested the relationship between GGT and all-cause and CV mortality in a population-based cohort of elderly individuals (n=1038) from the Invecchiare in Chianti study, which enrolled a random sample of people older than 65 years living in the Chianti area in Tuscany and followed up them for a median time of 9 years. In light of the pathophysiological relationship between GGT and oxLDL alluded to before (6), a pre-specified goal of the present study was testing whether oxLDL modifies the relationship between GGT, CV and all-cause mortality.

## METHODS

### *Study population*

The elderly InCHIANTI (13) population included 1155 participants aged between 65 and 102 years randomly selected from residents in the Chianti geographic area. The baseline data collection started in September 1998 and lasted until March 2000;

thereafter, participants were fully re-evaluated every 3 years and the fourth follow-up is still ongoing.

The present study was performed on 1038 participants out of the 1155 original cohort because we excluded individuals with missing serum GGT (n=112), documented liver disease (n=4) or because of extremely high serum level of GGT (n=1) identified as a statistically significant outlier (GGT=565 U/L) by Grubbs' test (P<0.001).

Alcohol consumption was assessed by self-reported daily intake of wine, beer and spirits. The content of ethyl alcohol was calculated as follows: 5 g ethyl alcohol in 100 mL of beer, 13 g in 100 mL of wine and 50 g in 100 ml of spirits. In agreement with WHO Guidelines, 40 g/day ethyl alcohol for males and 20 g/day for females were taken as a cut-off for identifying heavy drinkers.

***Follow-up and incident study outcomes***

The primary outcomes were all-cause and CV mortality. After the enrolment, individuals were monitored for a median of 9 years (ranging from 0.15-10.5 years). CV deaths were classified following ICD9 diagnosis codes from 410 to 438.

***Laboratory measurements***

Serum GGT was measured through an enzymatic colorimetric assay using a Roche analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). The minimum detectable threshold was 3 U/L and the measure range was 3-1200 U/L. The intra-assay coefficient of variation (CV) was 1.5% and the inter-assay CV was 1.4%. The normal values considered for GGT were: 10-50 U/L in men and 10-38 U/L in women. Oxidized LDL was measured using an enzyme-linked immunoassay (ELISA) kit (Mercodia AB, Uppsale, Sweden). The intra-assay and the inter-assay CV was 6% and 5%, respectively. High sensitivity C-reactive protein (CRP) was measured by a colorimetric competitive immunoassay that uses purified protein and polyclonal anti-CRP antibodies. The minimum detectable threshold was 0.03 mg/L and the inter-assay CV was 5%. Homocysteine were measured by fluorimetric polarized immunoassay method (IMX, Abbott Laboratories). The minimum detectable threshold was 0.5 μmol/L and the inter-assay CV was 4.3%.

***Statistical analysis***

Cross-sectional data were analyzed by univariate and multiple linear, logistic and Cox regression analyses. Multiple models included GGT as well as traditional risk factors (age, gender, smoking, diabetes, LDL cholesterol and blood pressure), factors peculiar to liver disease (transaminases, alkaline phosphatase, alcohol consumption), BMI, hemoglobin, oxidized LDL, C-reactive protein (CRP), homocysteine and creatinine clearance. To account for over-fitting (i.e. when the number of covariates overcame 1 variable every 10 study outcomes) a shrinkage correction was applied to Cox and logistic regression models (14). A backward elimination strategy was applied to logistic regression analysis. Interaction analysis was performed by the standard linear combination method. The proportionality assumption was tested by analyzing the Schoenfeld residuals and no violation was found. The functional form of key-covariates (including interaction term) was investigated by the analysis of Martingale residuals and the use of both risk factor (GGT) and effect modifier (oxLDL) as continuous variables resulted to be the best functional form for capturing the risk of all-cause and CV mortality explained by this biomarker. To assess whether early deaths could affect the study results a sensitivity analysis excluding patients who died within the first year from the enrolment was carried out. Furthermore, to minimize the potential distortion of heavy drinking (> 40 g/day ethyl alcohol for males and 20 g/day for females) a sensitivity analysis excluding heavy drinkers was performed. Data were expressed as odds ratio (OR), hazard ratios (HR), 95% confidence intervals (CI) and P values, as appropriate. All analyses were performed by standard statistical packages (SPSS for Windows Version 9.0.1, 11 Mar-1999, Chicago, Illinois, USA; STATA/SE 9.0 StataCorp LP, TX, USA).

**RESULTS**

The main demographic and clinical characteristics of the study population are summarized in **Table 1**. The study cohort included 1038 subjects (43% males) with a median age of 74 years (inter-quartile range: 69-79). Biochemical parameters,

including liver enzymes, were in the normal range and the mean value and SD of oxLDL was 42±13 U/L.

**Table 1.** Main Demographic, Somatometric and Clinical Characteristics of the Study Population

| | N=1038 |
|---|---|
| Age (years) | 74 (69-79) |
| Males (%) | 454 (43) |
| Diabetes (%) | 114 (11) |
| Smoking (%) | 141 (14) |
| CV comorbidities (%) | 152 (15) |
| Alcohol consumption (g/day) | 8.8 (0-29.9) |
| BMI (kg/m$^2$) | 27 (15-30) |
| Systolic BP (mmHg) | 150±20 |
| Diastolic BP (mmHg) | 84±9 |
| Lipid-lowering agents | 45 (4) |
| Glucose (mg/dL) | 89 (81-100) |
| LDL cholesterol (mg/dL) | 136±34 |
| Haemoglobin (g/dL) | 13.7 ±1.4 |
| C reactive protein (μg/mL) | 2.8 (1.3-5.9) |
| Alkaline phosphatase (U/L) | 203 (168-246) |
| Homocysteine (μmol/L) | 14.5 (12.2-17.8) |
| GGT (U/L) | 19 (14-28) |
| AST (U/L) | 20 (17-23) |
| ALT (U/L) | 17 (13-22) |
| oxLDL (U/L) | 42±13 |
| Creatinine clearance (ml/min/1.73m$^2$) | 65±19 |

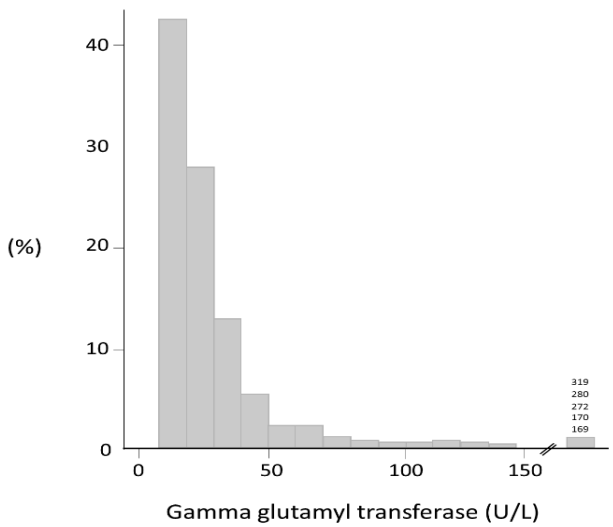
Past CV events were defined as the presence of at least one of the following documented comorbidities at enrolment: myocardial infarction, angina, peripheral vascular disease, stroke or coronary surgery/angioplasty.

Data are expressed as mean± SD, median and inter-quartile range or as percent frequency as appropriate. Abbreviations: BMI=body mass index; BP=blood pressure; LDL=low-density lipoproteins; GGT= gamma-glutamyltransferase; AST= aspartate aminotransferase; ALT=alanine aminotransferase; oxLDL= oxidized low-density lipoproteins.

***Clinical and functional correlates of GGT***

Serum levels of GGT had a left skewed distribution with a median value of 19 U/L (inter-quartile range: 14 to 28 U/L) (**Figure 1**).

**Figure 1.** Distribution of serum GGT levels in the study population




In a multiple linear regression model, including all univariate correlates of GGT, the independent correlates of this biomarker were ALT, male gender, C reactive protein, alcohol consumption, alkaline phosphatase, hemoglobin, CV comorbidities, oxLDL and homocysteine whereas AST, creatinine clearance, BMI, smoking, age, systolic BP, LDL cholesterol, diabetes and lipid lowering agents were not (P ranging from 0.28 to 0.99) (**Supplementary Table I**).

**Supplementary Table I.** Correlation matrix of the functional correlates of GGT and independent correlates of GGT

| | **Correlation matrix GGT versus** | | **Independent correlates of GGT GGT versus** | |
|---|---|---|---|---|
| Variables | r | P | Beta | P |
| Age | -0.07 | 0.03 | 0.03 | 0.38 |
| Male gender | 0.27 | <0.001 | 0.14 | <0.001 |
| Diabetes | 0.008 | 0.79 | -0.01 | 0.70 |
| Smoking | 0.09 | 0.003 | 0.008 | 0.77 |
| CV comorbidities | 0.14 | <0.001 | 0.09 | 0.001 |
| Alcohol consumption | 0.20 | <0.001 | 0.11 | <0.001 |
| BMI | 0.09 | 0.006 | 0.02 | 0.47 |
| Systolic BP | 0.02 | 0.56 | 0.03 | 0.28 |
| Diastolic BP | -0.01 | 0.74 | ….. | ……. |
| Lipid-lowering agents | 0.01 | 0.69 | 0.02 | 0.44 |
| Glucose | 0.16 | <0.001 | ….. | …… |
| LDL cholesterol | 0.03 | 0.35 | 0.03 | 0.31 |
| Haemoglobin | 0.23 | <0.001 | 0.10 | 0.003 |
| C reactive protein | 0.16 | <0.001 | 0.14 | <0.001 |
| Alkaline Phosphatase | 0.16 | <0.001 | 0.11 | <0.001 |
| Homocysteine | 0.07 | 0.02 | 0.06 | 0.03 |
| AST | 0.31 | <0.001 | -0.02 | 0.69 |
| ALT | 0.39 | <0.001 | 0.38 | <0.001 |
| oxLDL | 0.10 | 0.002 | 0.09 | 0.004 |
| Creatinine clearance | 0.13 | <0.001 | 0.001 | 0.99 |

Data are Pearson product moment correlation coefficients and P values. In the multiple linear regression model we introduced systolic BP (as an indicator of BP burden) instead of diastolic BP and diabetes instead of serum glucose. However, also forcing diastolic BP (instead of systolic BP) and serum glucose (instead of diabetes), the results remained materially unchanged.

***GGT and past CV events***

At baseline, 207 past CV events occurred in 152 individuals. In detail, myocardial infarction in 25 cases, coronary surgery/angioplasty in 16 cases, stroke in 36 cases, angina in 34 cases, peripheral vascular disease in 96 cases. In logistic regression analysis, GGT adjusted for age and sex was associated (P=0.03) with past CV events (OR:1.14, 95%CI:1.01-1.27). In a parsimonious backward logistic regression model adjusting for age, sex, CRP, alcohol consumption, ALT and diabetes the OR of a 20 U/L increase in GGT for the risk of past CV events was 1.23 (95%CI:1.06-1.43; P=0.01). Furthermore, the GGT-CV link was confirmed in a multiple logistic regression model [(OR:1.24, 95%CI:1.07-1.44; P=0.005); (shrinkage corrected OR:1.19, 95%CI:1.02-1.39;

P=0.02)] adjusting for the full list of traditional and non-traditional risk factors considered in this study (**Supplementary Table II**).

**Supplementary Table II.** Multiple logistic regression model of past CV events

| | Units of increase | Past CV disease | |
|---|---|---|---|
| | | Odds ratio (CI 95%) | P value |
| GGT | 20 U/L | 1.24 (1.07-1.44) | 0.005 |
| Age | 1 year | 1.03 (0.99-1.07) | 0.06 |
| Male gender | | 4.05 (2.54-6.46) | 0.001 |
| Current smokers | yes/no | 1.32 (0.79-2.20) | 0.28 |
| BMI | 1 $Kg/m^2$ | 0.97 (0.91-1.03) | 0.31 |
| LDL cholesterol | 1 mg/dl | 0.99 (0.99-1.01) | 0.38 |
| C reactive protein | 1 μg/mL | 1.02 (1.00-1.04) | 0.05 |
| Systolic blood pressure | 1 mmHg | 1.00 (0.99-1.01) | 0.94 |
| Alkaline phosphatase | 1 U/L | 1.00 (0.99-1.01) | 0.55 |
| Haemoglobin | 1 g/dL | 0.97 (0.84-1.13) | 0.72 |
| Alcohol consumption | 1 g/day | 0.99 (0.98-1.01) | 0.05 |
| AST | 1 U/L | 0.99 (0.95-1.03) | 0.55 |
| ALT | 1 U/L | 0.98 (0.95-1.01) | 0.18 |
| Diabetes | yes/no | 1.55 (0.90-2.67) | 0.11 |
| Creatinine clearance | 1 $ml/min/1.73m^2$ | 1.00 (0.99-1.02) | 0.78 |
| oxLDL | 1 U/L | 1.01 (0.99-1.03) | 0.10 |
| Homocysteine | μmol/L | 0.98 (0.95-1.01) | 0.18 |

Abbreviations: GGT= gamma-glutamyltransferase; BMI=body mass index; AST= aspartate aminotransferase; ALT=alanine aminotransferase; oxLDL=oxidized low-density lipoproteins

***GGT, all-cause and CV mortality***

During the follow-up period (median 9 years, range 0.15- 10.5 years), 401 individuals died, 168 of them (42%) for cardiovascular causes. In an age and sex adjusted Cox regression model, 20U/L increase in serum GGT signaled a parallel 10% increase in the risk of all-cause mortality (HR:1.10, 95%CI:1.03-1.18, P=0.007). In a multiple Cox regression analysis adjusting for the same set of variables applied in the multiple logistic regression model (**Supplementary Table III**), GGT was confirmed as an independent risk factor of mortality [HR (20 U/L increase):1.11, 95%CI:1.02-1.21, P=0.02]. Similarly, GGT predicted CV mortality both in age and sex adjusted model (HR:1.12, 95%CI:1.01-1.24; P=0.04) and in an analysis [(HR:1.17, 95%CI:1.03-1.34; P=0.02); shrinkage corrected HR:1.17, 95%CI: 1.02-1.33; P=0.02)] adjusting for the

same set of variables applied in the multiple logistic regression model. In sensitivity analyses (N=937) excluding patients who died for malignancies or within 1 year from the enrolment, the HR of GGT for all-cause [HR (20 U/L increase):1.13, 95%CI:1.02-1.25, P=0.02] and CV death (shrinkage corrected HR:1.17, 95%CI:1.03-1.34, P=0.02) remained the same.

**Supplementary Table III.** Multiple Cox regression models of all-cause and CV mortality

| | Units of increase | All-cause mortality | | CV mortality | |
|---|---|---|---|---|---|
| | | Hazard ratio (CI 95%) | P value | *Hazard ratio (CI 95%) | P value |
| GGT | 20 U/L | 1.11 (1.02-1.21) | P=0.02 | 1.17 (1.03-1.33) | 0.02 |
| Age | 1 year | 1.13 (1.11-1.15) | P<0.001 | 1.14 (1.11-1.17) | <0.001 |
| Male gender | | 1.26 (0.98-1.63) | P=0.08 | 1.55 (1.04-2.29) | 0.03 |
| Current smokers | yes/no | 1.93 (1.43-2.63) | P<0.001 | 1.22 (0.67-2.22) | 0.51 |
| BMI | 1 $Kg/m^2$ | 1.01 (0.98-1.04) | P=0.49 | 1.04 (0.99-1.09) | 0.10 |
| LDL cholesterol | 1 mg/dl | 0.99 (0.98-0.99) | P=0.007 | 1.00 (0.99-1.01) | 0.36 |
| C reactive protein | 1 μg/mL | 1.01 (1.01-1.02) | P=0.006 | 1.01 (1.00-1.02) | 0.02 |
| Systolic BP | 1 mmHg | 1.01 (0.99-1.01) | P=0.17 | 1.01 (0.99-1.01) | 0.10 |
| Alkaline phosphatase | 1 U/L | 1.00 (1.00-1.01) | P=0.01 | 1.00 (0.99-1.01) | 0.47 |
| Hemoglobin | 1 g/dL | 1.04 (0.96-1.13) | P=0.32 | 1.14 (1.01-1.30) | 0.04 |
| Alcohol consumption | 1 g/day | 0.99 (0.98-0.99) | P=0.03 | 0.98 (0.97-0.99) | <0.001 |
| AST | 1 U/L | 1.01 (0.99-1.04) | P=0.26 | 1.01 (0.97-1.04) | 0.79 |
| ALT | 1 U/L | 0.98 (0.96-0.99) | P=0.01 | 0.97 (0.95-1.01) | 0.11 |
| Diabetes | yes/no | 1.17 (0.85-1.61) | P=0.35 | 1.22 (0.73-2.03) | 0.45 |
| Creatinine clearance | 1ml/min/1.73$m^2$ | 1.00 (0.99-1.01) | P=0.80 | 0.99 (0.98-1.00) | 0.09 |
| oxLDL | 1 U/L | 1.01 (0.99-1.02) | P=0.18 | 0.99 (0.98-1.01) | 0.51 |
| Past CV events | yes/no | 1.48 (1.15-1.92) | P=0.002 | 1.37 (0.92-2.05) | 0.12 |
| Homocysteine | μmol/L | 1.02 (1.00-1.03) | P=0.002 | 1.02 (1.00-1.04) | 0.05 |

Abbreviations: GGT= gamma-glutamyltransferase; BMI=body mass index; LDL= low-density lipoproteins; AST= aspartate aminotransferase; ALT=alanine aminotransferase; oxLDL= oxidized low-density lipoproteins.
* Shrinkage corrected.

***GGT and clinical outcomes: effect modification by oxidized low-density lipoprotein***

Because oxLDL and GGT reflect reactive oxygen species burden, we hypothesized that coexistence of high oxLDL and GGT may amplify the risk for all-cause and CV mortality in the elderly population of the InCHIANTI study. Oxidized LDL per sé failed to show a meaningful link with all-cause and CV death (HR:0.99 for both outcomes). However, oxLDL amplified the effect of GGT on all-cause mortality both in age and sex adjusted Cox models (P for the effect modification=0.001) and adjusted analyses (P for interaction=0.003) (**Table 2**).

**Table 2.** Multiple Cox regression model of the oxLDL-GGT interaction for all-cause mortality

| | Units of increase | All-cause mortality | |
|---|---|---|---|
| | | Hazard ratio (CI 95%) | P value |
| GGT<br>oxLDL<br>GGT x oxLDL (*interaction term*) | 20 U/L<br>1 U/L<br>20 $U^2/L^2$ | **See Figure 1** | **P for interaction=0.003** |
| Age | 1 year | 1.13 (1.11-1.15) | 0.001 |
| Male gender | | 1.26 (0.98-1.63) | 0.07 |
| Current smokers | yes/no | 1.91 (1.41-2.59) | 0.001 |
| BMI | 1 $Kg/m^2$ | 1.01 (0.98-1.04) | 0.59 |
| LDL cholesterol | 1 mg/dl | 0.99 (0.98-0.99) | 0.007 |
| C reactive protein | 1 µg/mL | 1.01 (1.01-1.02) | 0.006 |
| Systolic blood pressure | 1 mmHg | 1.00 (0.99-1.01) | 0.26 |
| Alkaline phosphatase | 1 U/L | 1.00 (1.00-1.01) | 0.005 |
| Hemoglobin | 1 g/dL | 1.04 (0.96-1.12) | 0.34 |
| Alcohol consumption | 1 g/day | 0.99 (0.98-0.99) | 0.04 |
| AST | 1 U/L | 1.01 (0.99-1.04) | 0.21 |
| ALT | 1 U/L | 0.98 (0.96-0.99) | 0.02 |
| Diabetes | yes/no | 1.17 (0.85-1.62) | 0.33 |
| Creatinine clearance | 1 $ml/min/1.73m^2$ | 1.00 (0.99-1.01) | 0.70 |
| Past CV events | yes/no | 1.43 (1.11-1.85) | 0.006 |
| Homocysteine | µmol/L | 1.02 (1.01-1.03) | 0.002 |

Abbreviations: GGT= gamma-glutamyltransferase; oxLDL=oxidized low-density lipoproteins; BMI=body mass index; LDL=low-density lipoproteins; AST= aspartate aminotransferase; ALT=alanine aminotransferase

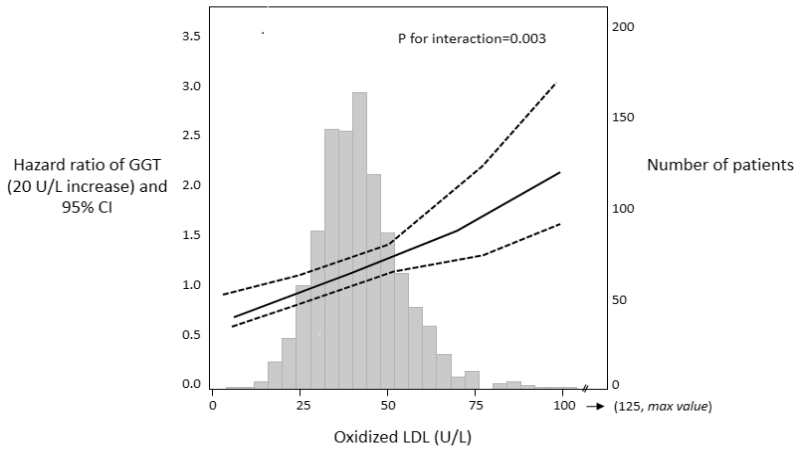



**Figure 2.** Effect modification of oxLDL levels on the GGT-mortality link (adjusted for age, gender, smoking, BMI, LDL cholesterol, C reactive protein, systolic BP, alkaline phosphatase, hemoglobin, alcohol consumption, AST, ALT, homocysteine, diabetes, creatinine clearance and past CV events). The hazard ratio for all-cause mortality portended by a fixed increase (20U/L) in serum GGT is reported on the left scale. The continuous line represents the shape of the HRs throughout oxLDL levels and the dotted lines the corresponding 95% CI. In the background the distribution of oxLDL is plotted and the number of patients corresponding to each column of the histogram is reported on the right scale.

As shown in **Figure 2,** the risk excess for all-cause mortality portended by a fixed increase in GGT (20 U/L) was progressively higher across increasing values of oxLDL.

A sensitivity analysis excluding individuals who were heavy drinkers (n=194), confirmed the oxLDL-GGT interaction for all-cause mortality (P<0.001). Of note, this interaction was specific because no similar effect modification existed for AST, ALT, CRP, smoking, alcohol consumption and other traditional or non-traditional risk factors (P ranging from 0.16 to 0.97).

An interaction analysis carried out to test the effect modification by oxLDL on the GGT-CV mortality link (**Supplementary Table IV**) showed a similar trend in age and sex adjusted Cox model (P for interaction=0.02) but this interaction just failed to achieve the formal statistical significance in both a fully adjusted analysis (P for interaction=0.08) and a sensitivity analysis (P=0.18) excluding heavy drinkers.

**Supplementary Table IV.** Multiple Cox regression model of the oxLDL- GGT interaction for CV mortality

| | Units of increase | CV mortality | |
|---|---|---|---|
| | | Hazard ratio (CI 95%) | P value |
| GGT<br>oxLDL<br>GGT x oxLDL (*interaction term*) | 20 U/L<br>1 U/L<br>20 $U^2/L^2$ | ***P for interaction=0.08** | |
| Age | 1 year | 1.15 (1.11-1.18) | 0.001 |
| Male gender | | 1.59 (1.07-2.36) | 0.02 |
| Current smokers | yes/no | 1.22 (0.67-2.20) | 0.52 |
| BMI | 1 $Kg/m^2$ | 1.04 (0.90-1.09) | 0.10 |
| LDL cholesterol | 1 mg/dl | 1.00 (0.99-1.01) | 0.35 |
| C reactive protein | 1 μg/mL | 1.01 (1.00-1.02) | 0.009 |
| Systolic blood pressure | 1 mmHg | 1.00 (0.99-1.01) | 0.10 |
| Alkaline phosphatase | 1 U/L | 1.00 (0.99-1.01) | 0.40 |
| Hemoglobin | 1 g/dL | 1.15 (1.02-1.30) | 0.03 |
| Alcohol consumption | 1 g/day | 0.98 (0.97-0.99) | <0.001 |
| AST | 1 U/L | 1.00 (0.97-1.04) | 0.69 |
| ALT | 1 U/L | 0.97 (0.94-1.01) | 0.10 |
| Diabetes | yes/no | 1.26 (0.75-2.09) | 0.38 |
| Creatinine clearance | 1 ml/min/1.73$m^2$ | 0.99 (0.98-1.01) | 0.08 |
| Past CV events | yes/no | 1.34 (0.89-2.00) | 0.16 |
| Homocysteine | μmol/L | 1.02 (1.00-1.04) | 0.04 |

*Given the fact that the interaction did not achieve statistical significance (P=0.08), no shrinkage correction was adopted.

**DISCUSSION**

In this cohort study conducted in older persons living in the Chianti area of Italy, serum GGT levels associated with history of CV disease and predicted the risk for all-cause and CV death independently of other risk factors, including liver disease and alcohol consumption. Furthermore, in a pre-specified interaction analysis circulating oxLDL amplified the effect of GGT on mortality.

Meta-analyses of studies in the general population and in high risk conditions (4, 5) coherently showed that GGT predicts an excess risk for death and fatal CV events. Importantly, the excess risk by GGT for these outcomes is largely independent of liver disease and alcohol consumption, i.e. the two major environmental factors responsible for raised GGT in human diseases. Of note the strength of the association between GGT and all-cause mortality was second only to that by age and CRP and the same outcome, further emphasising the relevance of non-traditional risk factors in the elderly (15). The vast majority of these studies were based on cohorts of young and middle-aged adults (4, 5). Until now just one community study specifically focused on an elderly population (the Rancho Bernardo study) (8). In this elderly population, GGT emerged as an independent predictor of all-cause and CV death. This observation contrasts with age-stratified analyses in the Minnesota Heart Survey where GGT was unrelated to CV death in people older than 70 years (9). Similarly, GGT failed to predict CV mortality in men older than 55 years in the British Regional Heart study cohort (12). Remarkably, an age-dependent attenuation of the health risk signalled by GGT was registered not only for the independent risk of CV death (16) but also for the incident risk of cancer (17). The age-dependent attenuation of the risk by GGT on major clinical outcomes suggests that the duration of exposure to this risk factor is critical to explain its link with adverse outcomes. In other words, shorter life expectancy in elderly people and competing risks by other diseases may prevent capturing any underlying link between GGT and mortality or CV disease in the elderly.

The InCHIANTI study is based on a cohort of prevalently healthy elderly people. Life expectancy in Tuscany (85 years for women and 80 years for men) is among the

longest worldwide and the InCHIANTI study has a quite long follow up with a median time of observation of 9 years. In this prevalently healthy cohort in subjects free of liver disease at baseline, GGT emerged as coherent predictor of death and fatal CV events independently of traditional (Framingham) and non-traditional risk factors including alcohol intake as well as CRP (18) and homocysteine (19), two established predictors of adverse outcomes in the elderly. High GGT is considered as a major biomarker of non-alcoholic fatty liver disease (NAFLD) (20) which is seen as a manifestation of metabolic syndrome (21). However, both in our study and in the Rancho Bernardo study (8) the link between GGT and mortality was largely independent of BMI and other variables underlying the metabolic syndrome suggesting that the independent risk of GGT for adverse clinical outcomes may underlie mechanism other than the metabolic syndrome.

GGT plays a crucial role in oxidative processes favouring the cellular supply of glutathione (GSH), the major thiol antioxidant in human body (2). GGT is ubiquitously expressed to the cell-surface where it promotes the extracellular catabolism of GSH, allowing for precursor amino acids to be internalized and reused for intracellular GSH synthesis in a continuous "GSH cycling" across the plasma membrane (22). Accordingly, GGT associates directly with F2-isoprostanes, an established marker of oxidative stress (23) and inversely with serum antioxidants (24, 25). On the other hand, experimental data exists indicating that GGT may per sé trigger the production of ROS via a sulphur di-aminoacid (cysteinyl-glycine) generated from GSH hydrolysis (22, 26).

Because GGT in atherosclerotic plaques co-localizes with oxLDL, this co-localization may be critical in the pathogenesis of atherosclerosis (6), a possibility supported by the observation that circulating GGT is bound to LDL (25). In a pre-specified interaction analysis we found an effect modification by oxLDL for the risk of death predicted by GGT levels. We observed a similar interaction for fatal CV events but, perhaps due to the relatively limited number of events, this effect just failed to achieve statistical significance (P=0.08). Such an interaction suggests that GGT levels may underlie a

mechanism which amplifies the toxic effects of oxLDL on the vascular system or vice versa.

In conclusion, in this cohort study in a population of relatively healthy elderly people, GGT is directly associated with the incident risk of all-cause and CV death independently of a large set of potential confounders and circulating oxLDL amplifies the effect of GGT on all-cause but not CV mortality in older adults. This study supports the contention that GGT may have a role in oxidative stress-mediated adverse health outcomes in the elderly.

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**CONFLICT OF INTEREST**

All the authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

*Belinda Spoto*: conceived the study and wrote the paper with Carmine Zoccali, and performed statistical data analysis with Graziella D'Arrigo and Giovanni Tripepi.

*Francesco Mattace-Raso*: critically revised the manuscript and provided significant intellectual contribution.

*Eric Sijbrands*: critically revised the manuscript and provided significant intellectual contribution.

*Graziella D'Arrigo*: performed statistical analysis and interpreted the results

*Giovanni Tripepi*: performed statistical analysis and interpreted the results

*Stefano Volpato*: critically revised the manuscript, provided significant intellectual contribution and was co-responsible of data handling/collection.

*Stefania Bandinelli*: critically revised the manuscript, provided significant intellectual contribution and was co-responsible of data handling/collection.

*Luigi Ferrucci*: supervised data collection and management, critically revised the manuscript and provided significant intellectual contribution.

*Carmine Zoccali*: conceived the study and wrote the paper with Belinda Spoto. Critically revised the manuscript and provided significant intellectual contribution.

# CHAPTER 8

Discussion

## DISCUSSION

Studies included in this thesis document that insulin resistance, inflammation and oxidative stress are crucial pathophysiological pathways that affect survival and CV integrity in high-risk populations. Observations from cross-sectional studies are supported by prospective evidence that gives consistent insights into the role of these detrimental processes in the high susceptibility to mortality and adverse CV outcomes in obese individuals and elderly subjects as well as in patients with CKD of various severity, representing a useful tool for risk stratification in these populations.

### Expression of inflammatory molecules in abdominal fat mass of obese individuals

Adipose tissue is distributed throughout the body in discrete fat compartments and the topography of its accumulation is relevant for the risk of adverse clinical outcomes. Adipose tissue is an abundant source of inflammatory cytokines and an excess of fat mass has been associated with a chronic subclinical inflammatory state. Central adiposity is more strongly associated with inflammation than peripheral adiposity (1) but a large waist encompasses both increased abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) not allowing to assess the specific contribution of these two fat compartments to obesity-related inflammation. By comparing the expression profiles of 19 major pro-inflammatory and 7 anti-inflammatory genes in SAT and VAT of severely obese patients, I found that these two fat compartments express the same set of inflammatory cytokines and that the large majority of pro-inflammatory genes is more expressed in SAT than in VAT, suggesting a stronger contribution of the subcutaneous fat compartment to the low-grade obesity-related inflammation. This evidence seems at odds with the traditional view of VAT as the main determinant of risk excess for adverse clinical complications and SAT as neutral or even protective component as for the same risk. However, the predominant component of fat mass in central adiposity is subcutaneous rather than visceral (2), being this latter just a minor segment of total fat depots equal to less than 1/5 of whole body fat tissue (1, 3). In addition, our findings are consistent with previous

evidence showing higher gene expression of TNFα, IL6 and IL1β (4-6) in SAT of obese patients rather than in VAT. I also found an upregulated expression of other two important pro-inflammatory molecules in SAT: SAA1 and PAI, which are responsible for the early response to injury and the inhibition of the fibrinolytic system, respectively. Moreover, I was able as first to demonstrate that IL8 is remarkably upregulated in SAT and that this increase was paralleled by an increase of the gene expression of the two IL8 receptors, pointing to an augmented IL8 signalling in SAT of obese individuals. In my study, IL18 was the sole pro-inflammatory cytokine showing a reverse expression pattern being upregulated in VAT. However, IL18 is a pleiotropic molecule promoting inflammation as well as the synthesis of anti-inflammatory cytokines such as IL10 (7). Of note, IL18-deficiency in mice causes hyperphagia, obesity, diabetes and atherosclerosis by massive fat deposition in the arterial walls (8). Thus, IL18 downregulation in SAT is in keeping with the hypothesis that the overall expression profile of cytokines in SAT denotes a more pro-atherogenic, metabolically adverse attitude.

Likewise, leptin and resistin, two potent pro-inflammatory adipokines, are upregulated in SAT as well as adiponectin which is the major anti-inflammatory protein secreted by adipose tissue. In support of an increased pro-inflammatory gene expression of SAT compared to VAT, I found that macrophage infiltration was comparable in the two fat compartments. However, a strong and positive correlation between SAT and VAT gene expression for the majority of the pro-inflammatory genes studied indicate that, though at different rates, the two fat compartments undergo similar changes in the expression profile of inflammatory cytokines. Interestingly, although cytokines secreted by adipose tissue usually act as autacoids, I found a strong association between the gene expression and the corresponding plasma levels for 4 inflammatory cytokines (IL8, IL18, SAA1, adiponectin) providing evidence that adipose tissue may contribute to circulating cytokine levels.

**TIMP-1, insulin resistance and cardiomyopathy in patients with kidney failure**

Left ventricular hypertrophy (LVH) and insulin resistance (IR) are pervasive complications of kidney failure (9, 10). LVH is characterized by hypertrophy of cardiomyocytes and abnormal accumulation of fibrous tissue in the interstitium of the myocardium (11). Ultrasonic studies *in vivo* in the myocardium of patients with kidney failure confirmed that fibrosis is a hallmark in LVH in this population (12, 13). There is consistent evidence that IR promotes myocardial fibrosis (14) by altering the balance between the synthesis and breakdown of extracellular matrix proteins (15). Indeed, raised insulin secretion shifts this balance toward reduction of extracellular matrix degradation via the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase Akt pathway (16). The fibrogenic process is regulated by matrix metalloproteinases (MMPs) that are a family of enzymes which degrade matrix proteins in the extracellular environment. The proteolytic activity of MMPs is modulated by a group of endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (17). The balance between MMPs and TIMPs is critical for the eventual remodeling of the myocardial tissue. TIMP-1 is currently considered a promising marker of fibrosis (18, 19). High circulating levels of TIMP-1 have been associated with LVH in both the general population (20) and in hypertensive patients (21, 22) and, in experimental models of pressure overload, over-expression of this biomarker goes in parallel with an increased LV mass (23). I have shown that a polymorphism in a genetic marker, the ENPP1 (i.e. ectonucleotide pyrophosphatase/phosphodiesterase 1) gene, of IR modifies the relationship between the pro-fibrotic TIMP-1 and LV geometry and function in a population of dialysis patients. The ENPP1 gene codes for a membrane glycoprotein that alters the intracellular insulin signalling by inhibiting insulin receptor autophosphorylation (24). In a recent study in the same set of patients with kidney failure, I documented a strong and significant association between the rs1974201 polymorphism in the ENPP1 gene and LV hypertrophy and concentric remodelling (25). Interestingly, in the latter study the ENPP1 rs1974201 polymorphism showed also a parallel association with insulin and glucose levels (46), thus providing evidence

of a direct involvement of carbohydrate metabolism in the pathogenesis of cardiomyopathy in dialysis patients. In this frame, I speculated that this polymorphism, which contributes to insulin resistance, can also modify the link between TIMP-1 and LV geometry and function. In the whole study population, TIMP-1 was directly and significantly (P=0.014) associated to E/A ratio, a marker of LV function, but unrelated to LV geometry as assessed by relative wall thickness (RWT) and LV mass-to-volume ratio. However, a separate analysis on stratified patients according to ENPP1 rs1974201 genotypes, shows that homozygous patients for the G allele exhibit a direct association of TIMP-1 with LV dysfunction but also a significant relationship between the same biomarker and the echocardiographic indices RWT and LV mass-to-volume ratio that increase of 22% and 14% respectively for a fixed increase in TIMP-1 levels (100 ng/ml). This finding points to the rs1974201 polymorphism as an effect modifier of the TIMP1-LV geometry and function. This is consistent with the hypothesis that the ENPP1 gene and IR are relevant players in the pathogenesis of concentric LVH as well as in the development of myocardial fibrosis in patients with kidney failure.

**The causal association between inflammation and CV complications in CKD: a Mendelian randomization study**

CKD is a clinical condition characterized by high CV risk and chronic systemic inflammation (26, 27). Although inflammation and CV disease are closely associated, being inflammation a critical component of the atherosclerosis process (28), the nature (casual vs non-causal) of this link remains elusive in CKD patients. Observational studies, indeed, are methodologically vulnerable to test causality because of potential bias and confounding. In this frame, the Mendelian randomization approach, by exploiting functional genetic polymorphisms as indicators of the effect of modifiable environmental exposures on disease, may offer the opportunity to overcome problems inherent to non-experimental studies. The categorization of patients according to pertinent alleles, in fact, generates a sort of

"natural" randomization which mimics the effects of a randomized clinical trial. By using this approach, I reported a strong and significant association between inflammation and adverse CV events in CKD patients and, notably, offered strong support to the hypothesis that this link is causal in nature, this finding being relevant because of the presence of inflammation at all CKD stages (29). IL-6 is an orchestrator of the inflammatory response and high circulating levels of this cytokine are predictive of mortality and CV events in several populations (30, 31), including dialysis patients (32-36). I used a functional polymorphism, i.e. the-174 G/C, in the promoter region of the IL-6 gene, as a reliable research tool for testing the nature (causal *vs* non causal) of the association between inflammation and CV complications in a population of patients with CKD of various severity. The -174 G/C is a single nucleotide polymorphism (SNP) which regulates circulating levels of IL-6 by modulating the rate of IL-6 gene transcription (37-43). In keeping with previous studies in patients with CV complications (37-41, 43), I found that, by stratifing the study population according to the -174 G/C polymorphism, CKD patients with CC genotype had higher circulating levels of IL-6 than those harboring GC or GG genotypes. Interestingly, I found that this polymorphism was independently associated with the history of CVD as well as with incident CV events and such associations went along with the relationships between serum IL-6 and the same outcomes in our cohort, specifically legitimating the use of this genetic marker as an unbiased means for assessing the causal nature of the link between the gene product (IL-6) of this polymorphism and the CV complications in CKD. Further, the observation that in our patients the -174 G/C polymorphism was not in Hardy-Weinberg equilibrium and that the frequency of the C allele was significantly higher in CKD patients than in the general population from the same geographical area, offers additional circumstantial evidence that IL-6 is a causal risk factor for CV events in CKD (44).

To the best of my knowledge, this was the first study which tested the association between IL-6 and CV events and investigated the causality of this link in a population of CKD patients not in dialysis. The only other study dealing with this issue was, in fact,

carried out by Barreto *et al*. (45) in a small CKD cohort (n=125) characterized by a quite limited number of CV events (n=22) and including 34% of dialysis patients. In addition, in the study by Barreto, the nature of the association between inflammation and CV outcomes was not tested because being observational in nature and based only on circulating IL-6, this study remains merely hypothesis generating thus leaving unresolved the critical question whether IL-6 is causally implicated in CV complications in CKD patients. Although, this issue demands specific experimental evidence (i.e. a formal randomized clinical trial), our Mendelian randomization study supports causal interpretations. As for the potential limitations to Mendelian randomization application, it is reasonable to believe that in our study all the assumptions required to consider this approach as an effective analysis strategy to infer causality in observational setting, are fulfilled. In fact, the -174G/C SNP is a functional polymorphism directly responsible for serum levels of IL-6 (37-43), our population is genetically homogeneous (46) and pleiotropy seems highly unlikely because of the location of the polymorphism in the promoter region of the gene. Remarkably, in line with our findings, meta-analytic data from genetic consortia exploring the effect of a polymorphism in the interleukin-6 receptor (IL6R) on the risk of coronary artery disease, showed that the allele which attenuated IL-6 signaling was significantly associated with reduced risk of coronary heart disease (46). Finally, a meta-analysis of clinical trials testing a monoclonal antibody against the IL6R (Tocilizumab) in rheumatoid arthritis documented that lowering serum levels of IL-6 is an effective strategy to induce the remission of this chronic inflammatory disease (47).

**Fat-mass and obesity-associated (FTO) gene and mortality in CKD**

About half of the fat mass variations is explained by genetic factors (48) and, among the large series of genes associated to human obesity, the FTO (fat-mass and obesity-associated) gene is of particular interest to investigate the complex relationship between high body mass index (BMI) and survival. This link deserves particular attention in CKD patients because, unlike the general population where excess

adiposity is directly and linearly associated with the risk of death, in CKD the relationship between obesity and mortality is U shaped or inverse, suggesting a protective effect of a high body fat mass in this population. Herein, I report an association between a polymorphism in the FTO gene and all-cause mortality in patients with CKD of various severity. I selected 17 tag SNPs that reflected the haploblock structure of the gene and captured the majority of the gene variability and explored their potential association with mortality in a cohort (the CREED-1 cohort) of dialysis patients. Among these polymorphisms, the rs708259 on intron 8 of the FTO gene resulted to be significantly associated with death. Specifically, in the CREED-1 cohort, patients with the A allele of the rs708259 polymorphism showed a 52% higher risk of mortality than patients without this allele. When I extended the analysis to other two dialysis cohorts, the North Apulian and the CREED-2 cohort, including patients comparable to those of the CREED-1 cohort as for demographic and clinical characteristics, I observed again excess mortality (+23% and + 21%, respectively) in A-allele carriers. Interestingly, I found such an association also in a third independent cohort of stage 2-5 CKD patients where A-allele carriers had an almost double risk of mortality relative to patients without A allele. While the association between the rs708259 polymorphism and mortality in my patients is statistically robust, the functional significance of this polymorphism is unknown. Because the rs708259 polymorphism is unrelated to BMI and diabetes in my patients, it seems unlikely that the FTO gene affects survival through its impact on mechanisms regulating energy balance or glucose metabolism. Interestingly, despite the well-known association of the FTO gene with obesity (49-53) and type 2 diabetes (49, 54-55), polymorphisms in this gene associate also with other major clinical conditions including cancer (56-58), hypertension (59), Alzheimer's disease (60) and kidney failure (61). Functionally, this gene codes for an enzyme that demethylates single-stranded DNA (62) and, thus, I speculate that this polymorphism may modulate relevant epigenetic modifications of other genes regulating various biological processes. Alternatively, the rs708259 polymorphism may exert its functional effects by modifying the bioavailability of the

transcript and/or the protein product of FTO but functional studies in appropriate models are needed to mechanistically interpret the association between this polymorphism and mortality in patients with CKD.

**The mutual relationship between resistin and adiponectin on all-cause and CV mortality in ESKD patients**

Resistin is a cysteine rich molecule synthesized in the adipose tissue (63, 64) and high levels of this peptide go along with inflammation and insulin resistance both in experimental models (63, 65-66) and in man (67,68). Specifically, adipocytes are considered the sole source of resistin in mice (63, 64) while in man this adipokine is expressed primarily in macrophages and its levels correlate more closely with inflammation than with insulin resistance (70-72). Plasma resistin is markedly elevated in ESKD (73, 74) but it is still debated if this phenomenon is a mere consequence of accumulation secondary to reduced renal clearance or if it mainly reflects chronic inflammation (74). In line with this evidence, in a population of ESKD patients, I found that resistin was not only higher than in normal subjects but also significantly related with serum level of C reactive protein, a recognized marker of inflammation, while it did not correlate with insulin resistance as assessed by circulating levels of insulin and HOMA-IR index.

Since resistin induces the expression of crucial pro-atherosclerotic mediators (75, 76), its role in CV risk was repeatedly questioned but evidence acquired so far is conflicting. Plasma resistin levels have been associated with coronary artery calcification (69), CV events and all-cause mortality in type 2 diabetes patients (77) as well as with major CV complications in the general population (78). However, no association between resistin and mortality was reported in patients with coronary artery disease (79). ESKD patients are a population with exceedingly high risk for death and CV events (80), thus testing the hypothesis that resistin is implicated in the high CV risk of these patients is a relevant question. Our study is the very first investigating the relationship between resistin and major clinical outcomes in this population. In this respect, I found an

association between resistin and all-cause and CV mortality that was statistically strong and quantitatively of potential clinical relevance. However, the effect of resistin on study outcomes was closely dependent on concurrent ADPN levels, being apparent and highly significant only in patients with low ADPN. Indeed, by a fixed increase in plasma resistin, there was a consistent excess risk for death and fatal CV events in patients in the first ADPN tertile whereas no risk excess for these outcomes was apparent in patients in the third ADPN tertile. This interaction being fully independent of potential confounders and in line with the evidence that adipose tissue cytokines have mutual inter-relationships that contribute to determine death and CV complications (81). The effect of ADPN on the relationship between resistin and all-cause and CV mortality has biological plausibility because ADPN and resistin have opposite effects on endothelial function and atherosclerotic process: resistin is a noxious factor for the CV system while ADPN is protective. Therefore, in ESKD patients, high ADPN reduces the CV risk excess sustained by high resistin levels.

**The role of the pro-oxidant GGT on survival in an Italian elderly population**

Gamma-glutamyltransferase (GGT) is a biomarker of liver disease (82, 83) but recent evidence supports an involvement of this enzyme in oxidative stress (84, 85), thus suggesting a potential role of GGT in the pathogenesis of CV disease (86). Since aging is a process heavely influenced by oxidative stress (87), eldelrly individuals provide a unique opportunity to study the relationship between GGT and CV outcomes. In this population-based cohort study conducted in people older than 65 years living in the Chianti area (Tuscany region) of Italy, I found that serum GGT levels associated with history of CV disease and predicted the risk for all-cause and CV death independently of a large set of risk factors, including liver disease and alcohol consumption, which are the two major environmental factors responsible for raised GGT levels in human diseases. These results are in line with meta-analyses data showing that GGT predicts an excess risk for death and fatal CV events in the general population and in patients with coronary artery disease and type 2 diabetes (82, 83). However, almost all the

studies included in the meta-analyses were carried out in cohorts of young and middle-aged adults (82, 83). Similarly, in the only community study (the Rancho Bernardo study) specifically focused on an elderly population (88), GGT emerged as an independent predictor of all-cause and CV death. In contrast, in an age-stratified analyses in the Minnesota Heart Survey, GGT resulted to be unrelated to CV death in people older than 70 years (89) and such a result was observed in the British Regional Heart study cohort where GGT failed to predict CV mortality in men older than 55 years (90). Remarkably, an age-dependent attenuation of the health risk signalled by GGT was registered not only for the risk of CV death (91) but also for the risk of cancer (92). The age-dependent attenuation of the risk by GGT on major clinical outcomes suggests that the duration of exposure to this risk factor is critical to explain its link with adverse outcomes. In other words, the short life expectancy of elderly people and competing risks by other diseases may prevent identifying an underlying link between GGT and mortality or CV disease in the elderly. The InCHIANTI study is based on a cohort of prevalently healthy elderly people. Life expectancy in Tuscany (85 years for women and 80 years for men) is among the longest worldwide and the InCHIANTI study has a quite long follow up with a median time of observation of 9 years. In this cohort with subjects free of liver disease at baseline, GGT emerged as coherent predictor of death and fatal CV events independently of other risk factors including alcohol intake. High GGT is a major biomarker of non-alcoholic fatty liver disease (NAFLD) (93), which may be considered as the hepatic manifestation of metabolic syndrome (94). However, both in our study and in the Rancho Bernardo study (88), the link between GGT and mortality was largely independent of BMI and other variables underlying the metabolic syndrome, suggesting that GGT may induce adverse clinical outcomes via mechanism(s) other than the metabolic syndrome.

GGT is ubiquitously expressed on the cell-surface where it promotes the extracellular catabolism of glutathione (GSH), allowing for precursor amino acids to be internalized and reused for intracellular GSH synthesis in a continuous “GSH cycling” across the plasma membrane (95). By favouring the cellular supply of glutathione (GSH), which is

a crucial antioxidant in human body (84), GGT plays a key role in oxidative processes. In addition, GGT may also trigger the production of ROS via a sulphur di-aminoacid (cysteinyl-glycine) generated from GSH hydrolysis (95, 96). Clinical evidence of the pro-oxidant action of GGT exists, indicating a direct correlation between GGT and F2-isoprostanes, an established marker of oxidative stress (97), and an inverse association of this hepatic enzyme with serum antioxidants (98, 99).

Recently, catalytically active GGT has been found within atherosclerotic coronary plaques (100) where it co-localizes with oxidized low-density lipoproteins (oxLDL). Serum GGT is partially adsorbed onto LDL which carry GGT inside the plaque (101), where this enzyme promotes the oxidation of LDL, likely contributing to oxidative events influencing plaque evolution and rupture (102). In light of the pathophysiological relationship between GGT and oxLDL, I observed that oxLDL modifies the risk of death predicted by GGT levels by amplifying the effect of GGT on all-cause and CV mortality in models controlling for potential confounders, suggesting that GGT levels may underlie a mechanism which amplifies the toxic effects of oxLDL on the vascular system or vice versa.

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# CHAPTER 9

## Summary

**SUMMARY**

In this thesis, epidemiologic and genetic data analyses in high-risk populations for cardiovascular disease (CVD) and excess mortality show that insulin resistance, inflammation and oxidative stress are crucial pathophysiological pathways mediating the increased susceptibility to adverse cardiovascular (CV) outcomes and mortality.

Specifically, findings from the cross-sectional study in severely obese subjects point to a prevailing role of the abdominal subcutaneous adipose tissue (SAT) on visceral adipose tissue (VAT) with respect to inflammatory gene expression, generating the hypothesis that topography of fat accumulation is relevant for the risk of inflammation and, consequently, of CV complications. Furthermore, a polymorphism in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene, a genetic marker of insulin resistance associated with myocardial hypertrophy and LV concentric remodeling in dialysis patients, modifies the relationship between a myocardial pro-fibrotic cytokine, the tissue inhibitors of metalloproteinase 1 (TIMP-1), and left ventricular (LV) geometry and function.

The crucial role of fat mass in the increased risk of adverse and/or fatal clinical events has also been prospectively investigated in both chronic kidney disease (CKD) and end-stage kidney failure (ESKF). In relation to the endocrine function of the adipose tissue, a study in a cohort of patients with kidney failure shows a mutual relationship between resistin and adiponectin (ADPN) in determining death and CV events. Indeed, the risk for all-cause and CV mortality portended by a fixed increase in plasma resistin depends on plasma ADPN concentration, being evident in patients with low levels of ADPN but absent in those with high levels of this adipokine. In line with this evidence, the variability of the fat mass and obesity-associated (FTO) gene, a genetic biomarker of diabetes and hypertension, contributed to mortality in three cohorts of patients with CKD.

In the context of inflammation as a trigger of CV complications, prospective follow up of patients with CKD revealed a strong and significant association between interleukin-6 (IL-6) and fatal and non-fatal CV events. Since serum IL-6 levels are genetically

regulated, it was obvious to use a Mendelian randomization method that exploited a functional polymorphism in the IL-6 gene as instrumental variable: my findings strongly support that the link between serum IL-6 levels and CV complications is causal in nature.

Finally, the role of oxidative stress on CV risk and mortality has been investigated in a cohort of healthy elderly since aging is, par excellence, the result of unbalanced pro-oxidant processes. In the participants of the Invecchiare in Chianti study, gamma-glutamyltransferase (GGT), a multifaceted biomarker of oxidative stress, emerged as a risk factor for all-cause and cardiovascular (CV) mortality independently of liver disease and alcohol intake. In addition, circulating oxidized low-density lipoproteins (oxLDL) amplified the effect of GGT on the adverse health outcomes. This supports the notion that GGT may underlie a mechanism which enhances the toxic effects of oxLDL on the vascular system or, vice versa, oxLDL enhances atherogenic properties of GGT.

# CHAPTER 10

# Samenvatting

**SAMENVATTING**

In dit proefschrift laten epidemiologische en genetische analyses zien dat insuline resistentie, inflammatie en oxidatieve stress cruciale pathofysiologische paden zijn en een verhoogde gevoeligheid voor ongunstige cardiovasculaire (CV) uitkomsten en mortaliteit mediëren in hoog-risicopopulaties voor cardiovasculaire aandoeningen (CVZ) en overmatige mortaliteit.

In het bijzonder, laten de bevindingen van de cross-sectionele studie zien dat de rol van het abdominale subcutane vetweefsel (SAT) een belangrijkere rol heeft dan het visceraal vetweefsel (VAT) met betrekking tot inflammatoire genexpressie. Hierbij is de hypothese gegenereerd dat de lokalisatie van de vetophoping relevant is voor het risico van inflammatie en daarmee het risico op CV-complicaties. Daarnaast is gevonden dat een polymorfisme in het ectonucleotide pyrofosfatase / fosfodiesterase 1 (ENPP1) gen, een genetische marker van insulineresistentie geassocieerd met myocardiale hypertrofie en LV concentrische remodellering bij dialysepatiënten, de relatie tussen een myocardiaal pro-fibrotisch cytokine, de weefselremmers van metalloproteinase 1 (TIMP-1), en linker ventrikel (LV) geometrie en functie modificeert.

De cruciale rol van vetmassa in het verhoogde risico op nadelige en/of fatale klinische gebeurtenissen is tevens prospectief onderzocht bij zowel personen met chronische nierziekte (CKD) als eind stadium nierfalen (ESKF). Met betrekking tot de endocriene functie van het vetweefsel, toont een onderzoek in een cohort van patiënten met nierfalen een wederzijds verband tussen resistine en adiponectine (ADPN) bij het bepalen van overlijden en CV-events.

Het risico voor mortaliteit door alle oorzaken en de CV mortaliteit dat wordt weergegeven door een forse toename in plasma resistine en afhangt van de ADPN-concentratie in het plasma, is duidelijk bij patiënten met lage niveaus van ADPN maar afwezig bij patiënten met hoge niveaus van deze adipokine. In overeenstemming met dit bewijs, droeg de variabiliteit van het vetmassa- en het obesitas-geassocieerde (FTO) gen, een genetische biomarker van diabetes en hypertensie, bij aan de mortaliteit in drie cohorten van patiënten met CKD.

In de context van inflammatie als een trigger voor CV-complicaties, toonde de prospectieve follow-up van patiënten met CKD een sterke en significante associatie tussen interleukine-6 (IL-6) en fatale en niet-fatale CV-gebeurtenissen. Omdat serum IL-6 niveaus genetisch zijn gereguleerd, was het noodzakelijk om een Mendeliaanse randomisatiemethode te gebruiken die een functioneel polymorfisme in het IL-6-gen als instrumentele variabele exploiteerde: mijn bevindingen ondersteunen sterk dat de associatie tussen serum IL-6-niveaus en CV-complicaties causaal van aard is.

Ten slotte is de rol van oxidatieve stress op CV-risico en mortaliteit onderzocht in een cohort van gezonde ouderen, omdat veroudering bij uitstek het resultaat is van ongebalanceerde pro-oxidantprocessen. In de deelnemers aan de Invecchiare in Chianti-studie kwam gamma-glutamyltransferase (GGT), een veelzijdige biomarker van oxidatieve stress, naar voren als een risicofactor voor mortaliteit door alle oorzaken en CV mortaliteit onafhankelijk van leverziekte en alcoholinname. Bovendien versterkten circulerende geoxideerde lipoproteïnen met lage dichtheid (oxLDL) het effect van GGT op de ongunstige gezondheidsresultaten. Dit ondersteunt het idee dat GGT mogelijk ten grondslag ligt aan een mechanisme dat de toxische effecten van oxLDL op het vasculaire systeem vergroot of, vice versa, dat oxLDL de atherogene eigenschappen van GGT vergroot.

**PHD PORTFOLIO**

| **Name PhD Student** | **Period** |
|---|---|
| Belinda Gilda Spoto | 2012-2019 |
| **Erasmus MC Department** | **Promoters** |
| Internal Medicine | Prof. dr. F.U.S. Mattace-Raso<br>Prof. dr. E.J.G. Sijbrands |
| **Research School** | **Copromoter** |
| Cardiovascular Research School Erasmus University Rotterdam (COEUR) | Dr. G.L.Tripepi |

PhD Training

**Research skills**

- Biomedical English

**In-depth courses**

- Principles of Genetic Epidemiology Course - Erasmus University Medical Center. Rotterdam, 11-15 August 2014
- Design Conceptual Foundation of Epidemiologic Study Design - Erasmus University Medical Center. Rotterdam, 11-15 August 2014
- Mendelian Randomisation Course - Erasmus University Medical Center. Rotterdam, 14-16 May 2014
- Scientific Writing Academy - Mario Negri Institute. Bergamo, 23-30 September 2012
- NDT Course for reviewers-to-be – ERA-EDTA. Leiden, 14-15 June 2012

**Invited lectures and Seminars**

- Primary immunodeficiencies and vaccinations. The USERN 2018 Congress and Prize awarding festival. Reggio Calabria, 12 November 2018
- Epidemiology and genetics of the atypical hemolytic-uremic syndrome. Hospital of Reggio Calabria, Unit of Nephrology Dialysis and Transplantation. Reggio Calabria, 28 May 2018
- Confounding and assessment of causation in observational studies and the opportunities of Mendelian randomization strategy. University of Messina, Department of Clinical Pharmacology. Messina, 6 July 2015
- Obesity, mortality and cardiovascular events: the conundrum of the inverse epidemiology. Congress of the Italian Society of Nephro-Cardiology. Reggio Calabria, 25-26 March 2015
- The role of adipose tissue in the cardiovascular risk: new acquisitions and perspectives. Congress of the Italian Society of Nephrology. Catania, 9 October 2014

**International Conferences**

49° European Dialysis and Transplantation Association Congress. Paris, France, 23-27 May 2012
50° European Dialysis and Transplantation Association Congress. Istanbul, Turkey, 18-21 May 2013
51° European Dialysis and Transplantation Association Congress. Amsterdam, The Netherlands, 31 May- 3 June 2014
52° European Dialysis and Transplant Association Congress. London, United Kingdom, 28-31 May 2015
53° European Dialysis and Transplant Association Congress. Vienna, Austria, 21-24 May 2016
54° European Dialysis and Transplant Association Congress. Madrid, Spain, 3-6 June 2017
56° European Dialysis and Transplant Association Congress. Budapest, Hungary, 13-16 June 2019

**Other**

Reviewer for:
- PLOS One
- CJASN
- Journal of Nephrology
- International Brazilian Journal of Urology
- American Journal of Hypertension
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Belinda Gilda Spoto was born in Reggio Calabria (Italy) on January 29th 1970. In 1993 she graduated with honour in Biological Science at University of Pisa and in 2001 she obtained the specialization in Medical Genetics at University of Catania. In January 2012, she entered a PhD Programme in Cardiovascular genetics under the surveillance of Prof. dr. F. Mattace-Raso and Prof. dr. E.J. Sijebrands at the Department of Internal Medicine - Section of Geriatric Medicine - of the Erasmus University Medical Center of Rotterdam (The Netherlands). Currently she is full researcher at the National Council of Research, Institute of Clinical Physiology (CNR-IFC) of Reggio Calabria (Italy) where she works in the Laboratory of Molecular Genetics. Her areas of expertise are genetic association studies and gene expression analysis. In 2003 she became the Head of the Gene Expression Section within her scientific Unit. Her scientific investigations are mainly focused on the genetics of cardiovascular risk in chronic kidney disease. She is scientific referent of national and international projects.