

Common Sequence Variants in *CD163* Gene are Associated with Plasma Triglyceride and Total Cholesterol Levels in Severely Obese Individuals

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Abstract

Objective: The CD163 glycoprotein is a member of the scavenger receptor cysteine-rich superfamily acting as an inflammatory modulator inducing anti-inflammatory pathways. Previous findings from our group identified this gene as being differentially expressed in visceral adipose tissue (VAT) of severely obese men with vs. without the metabolic syndrome. The current study aimed to test the association between *CD163* gene polymorphisms and obesity-related metabolic complications.

Methods: Sequencing of the *CD163* gene region was conducted in 25 severely obese individuals. Eleven tagging SNPs (tSNP) were selected and tested for association with obesity-related complications in nearly 1900 severely obese individuals. To further explore potential mechanisms underlying associations identified, the impact of tSNPs on methylation levels of 3 CpG sites (two promoter and one intronic) and gene expression levels were tested in a subset of 14 individuals.

Results: Rare allele carriers for rs7980201 demonstrated lower fasting total cholesterol (total-C) levels ($p=0.01$) while rs4883263 rare allele carriers had increased total-C ($p=0.04$) and triglyceride (TG) levels ($p=0.01$). An association identified between rs7980201 SNP and methylation level of a promoter CpG site ($p=0.04$) suggested an impact on *CD163* gene methylation in VAT, but such association was not reflected at gene expression level.

Conclusion: The current study reports association of *CD163* gene variations with fasting total-C and TG levels and suggests that *CD163* SNPs could contribute to the inter-individual variability observed in obesity-related metabolic complications.

Keywords: Cardiometabolic risk; Cardiovascular diseases; Immunity; Inflammation; Visceral adipose tissue; Metabolic syndrome; Obesity

Introduction

Obesity increases the risk for several chronic diseases including cardiovascular diseases (CVD) and type 2 diabetes mellitus (T2DM) [1]. Excess accumulation of fat inside the abdominal cavity (visceral adipose tissue: VAT) is associated with altered insulin sensitivity, blood pressure (BP), and plasma lipid profile [2,3]. The clustering of many CVD risk factors including accumulation of abdominal fat, impaired glucose tolerance, dyslipidemia, and hypertension defines the metabolic syndrome (MetS) [4]. Increased circulating levels of inflammatory markers have been reported in obesity [5] concomitant to immune cell infiltration and activation in white adipose tissue [6] and have been identified as independent risk factors for CVD [7]. In addition, the number of macrophages in VAT has been positively associated with waist circumference and MetS [8,9].

The severely obese population is heterogeneous regarding CVD risk profile [10] and the pathogenesis of the MetS and its components in subgroups of obese individuals likely involves multiple interactions among behavioral, environmental, and genetic factors [11,12]. Our knowledge of the factors involved in the etiology of obesity-related metabolic disturbances remains largely incomplete, but evidence points toward adipose tissue dysfunction with a predominant role of immunity, inflammation and oxidative stress [13,14]. Genetic components were also shown to contribute to the development of these perturbations [15]. A previous transcriptomic study from our group comparing gene expression profile in VAT of non-diabetic obese men with the MetS (MetS+) vs. obese men without the MetS (MetS-) [16]

revealed *CD163* among the list of 489 differentially expressed genes. Being involved in the regulation of inflammation and oxidative stress (18) and being differentially expressed in VAT of MetS+ vs. MetS- (1.6-fold; $p = 0.02$), the *CD163* gene appeared as a candidate gene to test for association obesity-related complications.

The CD163 glycoprotein is a member of the scavenger receptor cysteine-rich superfamily exclusively expressed in monocytes/macrophages [17]. While various functions have been attributed to this protein [18], the most extensively studied function of CD163 is related to the regulation of inflammatory processes by induction of anti-inflammatory pathways [19] through the uptake of native haptoglobin (Hp) and haemoglobin (Hb) complexes [20,21]. Hp-Hb uptake upregulates heme oxygenase via autocrine mechanisms [22] consequently reducing oxidative stress and inflammation, and improving insulin sensitivity [19,23]. Increased level of *CD163* gene

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expression in VAT was reported in obese subjects compared to lean subjects [24]. Upon inflammatory activation of macrophages, the extracellular part of the CD163 receptor is cleaved to produce soluble CD163 (sCD163) which is released into blood [25,26]. Similar to membrane-bound CD163, increased concentrations of sCD163 have been measured in obese individuals [27-29]. Plasma levels of sCD163 were shown to be associated with adipose tissue *CD163* gene expression levels [30] as well as with diabetes and atherosclerosis [29-33].

Based on the physiological involvement of inflammation in the development of obesity-related metabolic complications and differential expression observed for *CD163* in VAT of MetS+ vs. MetS- obese individuals [16], we postulate that *CD163* gene variations may contribute to explain inter-individual variability observed in obesity-related metabolic complications in severely obese individuals. Associations between *CD163* polymorphisms and obesity-related metabolic complications were thus tested in a cohort of severely obese patients undergoing bariatric surgery. The impact of SNPs associated with obesity-related metabolic complications on gene methylation and expression levels was further analyzed to explore potential mechanisms underlying these associations.

Material and Methods

Patient selection

Between June 2000 and July 2012, 1883 severely obese Caucasian men (N=589) and women (N=1294) candidates for biliopancreatic diversion with duodenal switch at the Québec Heart and Lung Institute (Québec City, Québec, Canada) have been recruited. Specimens were obtained from the biobank of the Institute according to institutionally approved management modalities. All participants provided written informed consent before their inclusion. The surgical protocol is described elsewhere [34]. Waist girth, resting BP [systolic (SBP) and diastolic (DBP)], plasma lipid (total-cholesterol (total-C), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and triglycerides (TG)) and fasting glucose concentrations were measured preoperatively using standardized procedures [35]. Diabetes, hypertension, and dyslipidemia as well as medication were retrieved from medical files. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Despite it is not routinely measured on individuals from our biobank, plasma C-reactive protein (CRP) levels were obtained from a previous study for 1185 subjects [36] with a high-sensitivity CRP immunoassay using a monoclonal antibody coated with polystyrene particles. The assay was performed with a BN ProSpec nephelometer (Dade Behring, Mississauga, Ontario, Canada) according to the manufacturer's protocol. The presence of MetS was determined using the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATPIII) guidelines when an individual fulfilled three or more criteria [4].

Gene expression analysis

In a previous study [16], a subset of 14 obese men (BMI >40 kg/m²) not taking any medication to treat MetS features were selected. Half of this subset was considered affected by the MetS (MetS+ group, N=7) and were matched for age, BMI and smoking status to the other seven obese individuals without MetS (MetS- group, N=7). Gene expression profiling was then processed on those 14 individuals using oligonucleotide microarrays. Characteristics of these individuals, sorted by either MetS+ or MetS-, as well as procedures for expression profiling are available elsewhere [16]. Following data correction and normalization, the *CD163* gene, which is known to be implicated in

the immune/inflammatory response, was selected from the list of 489 differentially expressed genes in VAT of the MetS+ vs. MetS- subgroups.

CD163 gene expression was measured using real-time PCR (RT-PCR) (Applied Biosystems Gene Expression Assays; Applied Biosystems®, Foster City, CA, USA) in the same 14 omental adipose tissue samples selected for the original microarray experiment [16] in order to confirm initial microarray results. Primers and TaqMan® probes were obtained from Applied Biosystems (*CD163*: Hs00174705_m1, Applied Biosystems Foster City, CA, USA). Pre-designed primers overlapping the sixth and seventh coding exons were used. These samples were analyzed in triplicate and calibrated to the *GAPDH* housekeeping gene (endogenous control; *GAPDH*: Hs99999905_m1). Relative quantification estimations were achieved on an Applied Biosystems® 7500 Fast Real-Time PCR System in accordance with the manufacturer's recommendations and the $\Delta\Delta CT$ calculation method was used to evaluate the mean fold expression difference (MFED) between MetS- and MetS+ groups.

Sequencing and genotyping

Genomic DNA was extracted from the blood buffy coat of 25 severely obese individuals (MetS+=12; MetS-=13) including the 14 individuals selected for gene expression profiling using the GenElute Blood Genomic DNA kit (Sigma, St. Louis, MO, USA). The promoter region (~2100 bp from the beginning of exon 1), exons and intronic flanking regions of the *CD163* gene were first amplified and sequenced with primers designed using human *CD163* public sequence (accession number: NM_004244.5; contig number: NC_000012.12) to identify common variation. The BigDye® Terminator 3.1 kit was used for sequencing and samples were run on an ABI 3730/XL DNA Analyzer automated sequencer (Applied Biosystems). From the 20 SNPs identified by direct sequencing of the 25 severely obese men, 11 tagging single nucleotide polymorphisms (tSNP) (minor allele frequency \geq 5%; MAF) were selected using the Tagger selection algorithm of the Haploview software [37,38]. Selected tSNPs were genotyped in genomic DNA extracted from the blood buffy coat for the whole cohort using either custom or pre-designed primers and TaqMan probes (Applied Biosystems). Genotypes were determined using Applied Biosystems® 7500 Fast Real-Time PCR System and analyzed using ABI Prism SDS software version 1.2.3 (Applied Biosystems). The mean genotype call rate for those 11 SNPs in the whole cohort was 97.5%.

DNA methylation analysis

DNA methylation analysis was conducted on the 14 obese men initially selected from our cohort for gene expression profiling. Genomic DNA extraction was performed from 200 mg of VAT using the DNeasy Blood & Tissue kit (QIAGEN, Mississauga, Ontario, Canada). Bisulfite conversion and quantitative DNA methylation analysis were carried out from 1 μ g of DNA at the McGill University and Génome Québec Innovation Centre (Montreal, Canada). Infinium® HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA) were processed following the manufacturer's instructions. The BeadChip interrogates more than 485 000 methylation sites at a single-nucleotide resolution. Methylation data were visualized and analyzed with the GenomeStudio® software version 2011.1 (Illumina Inc.) and the Methylation Module. Methylation levels (beta values; β) were estimated as the ratio of signal intensity of the methylated alleles to the sum of methylated and unmethylated intensity signals of the alleles (β value=C/(T+C)). Data correction (background subtraction and normalization) was applied using internal control probe pairs. CpG sites with a detection p-value > 0.05 were not considered in the analysis. Methylation ratios (β values) of 3 CpG sites located within the *CD163*

locus and promoter region were extracted using the GenomeStudio Methylation Module.

Statistical analyses

Untransformed and unadjusted phenotypic data were reported as mean \pm SD. Gene expression microarray analysis was conducted using unpaired Student's *t*-test to identify differentially expressed genes between MetS+ and MetS- groups. The Haploview software [37] and the Tagger selection algorithm [38] were used to evaluate Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (r^2) and selection of tSNPs (MAF \geq 5%). Following genotyping in the whole cohort, rare homozygote genotypes showing genotype frequency below 5% were merged to heterozygote genotypes for statistical analysis (6 SNPs; rs79302630, rs11054195, rs4883263, rs7980201, rs76573683 and rs61729512). Phenotypic differences between genotype groups were tested using analysis of variance (general linear model, type III sum of squares) with adjustments for the effects of age, sex, BMI and medications to treat hypertension, dyslipidemia, or diabetes with the exception of BMI and waist girth which were adjusted for age and sex. Transformations were applied to non-normally distributed variables in order to meet the criteria for normality [logarithmic transformation (log 10): total-C/HDL-C ratio, fasting glucose and plasma CRP levels; negative inverse transformation ($1/(-1*(1 + X))$): HDL-C, TG]. Pairwise comparisons among genotype groups were performed using LS-means and Student's *t* tests when a genotype effect was identified. Genotype distribution of CVD risk factor-associated SNPs in the whole cohort according to the presence of metabolic syndrome were calculated for CVD risk factor-associated SNPs using Chi-square tests. Pairwise Pearson correlations between methylation and expression levels as well as between microarray and RT-PCR data were calculated. Statistical significance was defined as $p \leq 0.05$. P-values presented are for transformed and adjusted values when appropriate. SAS software version 9.3 (SAS Institute, Cary, NC) was used to perform the statistical analyses.

Results

Cohort description

The present study included 1883 severely obese individuals (589 men and 1294 women) undergoing bariatric surgery. From these

individuals, 1876 were successfully classified either as MetS+ (n=1527) or MetS- (n=349 or 20.6%). As shown in Table 1, this cohort of middle-aged, severely obese individuals demonstrates high mean fasting plasma glucose and TG levels, as well as elevated SBP according to the MetS criteria defined by the NCEP-ATPIII [4].

Gene expression in VAT

Gene expression microarray analysis in 14 severely obese men (MetS+, n=7; MetS-, n=7) previously revealed 489 differentially expressed genes between MetS+ and MetS- groups [16]. Although not listed at the time, the *CD163* gene was among the list of 489 differentially expressed genes and significantly overexpressed (1.6-fold; $p = 0.02$) in VAT of MetS+ vs. MetS- obese men. The same subset of individuals was used to validate microarray data using RT-PCR for the *CD163* gene. The *CD163* gene was found to be overexpressed in VAT of MetS+ obese men according to RT-PCR results (MFED = 1.51; $p=0.03$). *CD163* gene expression levels obtained by RT-PCR were strongly correlated to those obtained by microarrays ($r=0.69$, $p=0.007$).

SNPs identification and tSNPs selection

Sequencing of the promoter region, exons, and intronic flanking sequences of the *CD163* gene was conducted in a subset of 25 severely obese individuals and led to the identification of 20 gene variations (Table 2). From these SNPs, 19 were reported in the SNP database (dbSNP build 137) and 18 displayed a MAF \geq 5%. Rare SNP (MAF < 5%) rs116933274 (intron 3) was identified in both groups while one MetS+ individual was found to be heterozygote for c.3248-2A>G (intron 15). Among the 20 SNPs identified, 7 were located in the promoter and 7 were intronic. From the 6 remaining SNPs, 2 were located in exon 5, others were located in exons 2, 7 and 11, while the last one was located in the 3' untranslated region (exon 17). Exonic SNPs rs4883263 and rs61729512 resulted in amino acid changes p.Val342Ile and p.Thr901Met, respectively. Identification of tSNPs was carried out from the 18 SNPs with MAF \geq 5%. Eleven SNPs were selected and allowed to cover 94% of the sequence-derived genetic variability of the common polymorphisms (MAF \geq 5%) at the *CD163* locus. These 11 tSNPs were further genotyped in the whole cohort of 1883 severely obese individuals. The number of genotypes and HWE p-values obtained are shown in Table 3. One of these SNPs (rs61729512) displayed a deviation from HWE with an overrepresentation of rare homozygotes and has been excluded from analyses.

Variables	Number of subjects (N)	Means \pm SD
Number of subjects (% male)	1883	1883 (31.3%)
Age (years)	1871	43.1 \pm 10.6
BMI (kg/m ²)	1865	51.6 \pm 8.8
Waist girth (cm)	1865	149.3 \pm 17
Fasting glucose (mmol/l)	1860	6.6 \pm 2.3
Plasma lipid profile		
Total-C (mmol/l)	1871	4.68 \pm 0.96
LDL-C (mmol/l)	1853	2.66 \pm 0.84
HDL-C (mmol/l)	1869	1.23 \pm 0.35
TG (mmol/l)	1870	1.82 \pm 1.07
Total-C/HDL-C	1869	4.01 \pm 1.29
Blood pressure		
SBP (mm Hg)	1868	138.47 \pm 17.14
DBP (mm Hg)	1867	83.47 \pm 11.46
Plasma CRP (mg/L)	1185	11.04 \pm 9.12

Values are presented as mean \pm SD. Abbreviations: SD, standard deviation; BMI, body mass index; Total-C, total cholesterol; LDL-C; low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; CRP, C-reactive protein.

Table 1: Subjects' characteristics.

rs number	Localization ^a	Other designation ^b	Region	MAF	HWE p-values
rs7301572	chr12:7657543	c.-1257G>A	Promoter	0.36	1.00
rs10845306	chr12:7657427	c.-1141C>T	Promoter	0.16	1.00
rs79302630	chr12:7657399	c.-1113T>C	Promoter	0.06	1.00
rs11054197	chr12:7657322	c.-1036C>T	Promoter	0.10	1.00
rs28364798	chr12:7657121	c.-835delT	Promoter	0.30	0.71
rs10772428	chr12:7656902	c.-616G>A	Promoter	0.35	1.00
rs11054195	chr12:7656849	c.-563A>T	Promoter	0.08	0.24
rs3210140	chr12:7655137	c.70C>T	Exon 2	0.39	1.00
rs116933274	chr12:7653726	c.457+9T>A	Intron 3	0.04	1.00
rs4883264	chr12:7649653	c.855G>A	Exon 5	0.36	0.73
rs4883263	chr12:7649484	c.1024G>A; p.Val342Ile	Exon 5	0.12	0.57
rs7980201	chr12:7640589	c.1515G>A	Exon 7	0.10	1.00
rs62622400	chr12:7640336	c.1735+33C>T	Intron 7	0.06	1.00
rs76573683	chr12:7639054	c.2458+41A>C	Intron 10	0.06	1.00
rs10743939	chr12:7638020	c.2459-8C>T	Intron 10	0.30	1.00
rs61729512	chr12:7637769	c.2702C>T; p.Thr901Met	Exon 11	0.14	1.00
rs6488340	chr12:7635793	c.3088+170C>T	Intron 12	0.14	1.00
---	chr12:7633854	c.3248-2A>G	Intron 15	0.02	1.00
rs12304718	chr12:7632376	c.*9+80A>G	Intron 16	0.16	0.17
rs11054072	chr12:7623724	c.*279T>C	Exon 17	0.34	0.82

^a Position according to genome build 37. ^bReference sequences: NM_004244.5 and NP_004235.4. Abbreviations: MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

Table 2: *CD163* sequence variations identified by direct sequencing in 25 severely obese individuals.

SNPs	Number of genotypes	Common HMZ	HTZ	Rare HMZ	MAF	HWE p-values
rs10845306	1792	862	761	169	0.47	0.96
rs79302630	1867	1608	248	11	0.07	0.67
rs10772428	1806	867	769	170	0.31	0.98
rs11054195	1873	1721	148	4	0.04	0.66
rs4883264	1833	1045	678	110	0.24	1.00
rs4883263	1870	1764	102	4	0.03	0.05
rs7980201	1848	1513	320	15	0.09	0.67
rs76573683	1858	1609	239	10	0.07	0.73
rs10743939	1825	1046	680	99	0.24	0.40
rs61729512	1838	1384	386	68	0.14	0.00
rs11054072	1775	491	911	373	0.47	0.19

Abbreviations: SNP, single nucleotide polymorphism; HMZ, homozygotes; HTZ, heterozygotes; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

Table 3: Genotype distribution of selected tagging SNPs in the whole cohort.

Association of *CD163* SNPs

Associations between fasting plasma glucose, lipid profile, BP-related variables (SBP and DBP) and tSNPs were tested. Following adjustment for the effects of age, sex, BMI and medication, significant associations were identified for rs4883263 and rs7980201. Carriers of the rare allele for rs4883263 demonstrated elevated total-C ($p=0.04$) and TG ($p=0.01$) levels while carriers of the rare allele for rs7980201 demonstrated lower total-C levels than carriers of the wild-type allele ($p=0.01$; Table 4). A trend toward lower DBP ($p=0.08$; 83.5 ± 11.2 vs. 82.3 ± 11.3) was also observed in rare allele carriers for rs11054195 (Appendix Table A.1). Further analyses were conducted for CVD risk factor-associated SNPs (rs4883263 and rs7980201) to test differences in genotype distribution according to the presence or absence of MetS in the whole cohort. Genotype distribution for those two SNPs did not differ significantly between MetS+ and MetS- groups (Appendix Table A.2).

Gene methylation and expression analysis

To further explore the potential underlying mechanisms, associations between CVD risk factor-associated SNPs (rs4883263 and rs7980201), gene methylation and expression levels were tested in a subset of 14 individuals. Genotype distribution for these two SNPs

in this sample is provided in Appendix Table A.3. Methylation levels were available for 2 CpG sites located within the promoter region (1110 and 1193 bp upstream transcription start site) and one in intron 4. CpG sites with corresponding localization are provided in Table 5. Methylation levels of the cg07264679 CpG site located in promoter region was associated with rs7980201. Rare allele carriers showed lower methylation levels (86.6%) than homozygotes for common allele (90.0%). No association was found between genotypes and gene expression levels. In addition, methylation levels of the 3 CpG sites did not correlate with expression levels ($p=0.60$, 0.85 and 0.41 respectively for cg05939324, cg07264679 and cg03621974).

Discussion

Over the past years, *CD163* has raised attention for its function in inflammation and its role as a two-faced immunomodulator, stimulating or suppressing the immune response and inflammation in diverse conditions both under its membrane-bound and soluble forms [18]. The current study reports overexpression of *CD163* in VAT of severely obese individuals with the MetS, thus simultaneously extending the potential pathophysiological importance of this gene to obesity-related metabolic disease. These results are consistent with increased *CD163* expression level in VAT from obese subjects compared to lean subjects

Phenotypes	rs4883263		p-values ^b	rs7980201		p-values ^b
	Means ^a			Means ^a		
	Common HMZ	Rare allele carriers		Common HMZ	Rare allele carriers	
Number of subjects (N)	1764	106	---	1513	335	---
BMI (kg/m ²)	51.5 ± 8.5	51.2 ± 8.0	0.83	51.5 ± 8.6	51.1 ± 7.9	0.29
Waist girth (cm)	140.5 ± 17.6	139.2 ± 18.3	0.84	140.5 ± 17.8	139.9 ± 16.9	0.19
Fasting glucose (mM)	6.55 ± 2.34	6.63 ± 2.48	0.68	6.57 ± 2.37	6.51 ± 2.28	0.25
Lipid profile						
Total-C (mM)	4.66 ± 0.95	4.81 ± 1.03	0.04	4.69 ± 0.97	4.57 ± 0.88	0.01
LDL-C (mM)	2.65 ± 0.83	2.71 ± 0.81	0.25	2.67 ± 0.85	2.60 ± 0.77	0.14
HDL-C (mM)	1.23 ± 0.36	1.24 ± 0.32	0.62	1.24 ± 0.35	1.24 ± 0.39	0.34
TG (mM)	1.81 ± 1.04	2.02 ± 1.46	0.01	1.82 ± 1.00	1.76 ± 0.96	0.23
Total-C / HDL-C	4.00 ± 1.28	4.12 ± 1.47	0.11	4.02 ± 1.27	3.93 ± 1.34	0.38
Blood pressure						
SBP (mm Hg)	138.5 ± 16.8	137.1 ± 17.1	0.93	138.5 ± 17.1	137.5 ± 15.4	0.34
DBP (mm Hg)	83.3 ± 11.3	83.9 ± 9.8	0.66	83.4 ± 11.3	82.9 ± 10.6	0.32
CRP (mg/L)	10.81 ± 8.32 (N=1100)	9.90 ± 7.93 (N=68)	0.62	10.89 ± 8.57 (N=943)	10.44 ± 7.45 (N=209)	0.47

Boldface values represent statistically significant results ($P \leq 0.05$). ^aValues presented (means ± SD) are untransformed and unadjusted values. ^bP-values obtained are adjusted for the effect of age, sex, BMI and medication use except for BMI and waist girth which were adjusted for age and sex. Abbreviations: N, number; Total-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 4: Significant genotype differences identified in plasma fasting glucose and lipid levels and in blood pressure.

SNP	cg05939324	cg07264679	cg03621974	Expression
rs4883263	0.64	0.84	0.57	0.37
rs7980201	0.47	0.04	0.16	0.22

P-values for associations were obtained from a subset of 14 obese men (7 MetS+ and 7 MetS-). CpG sites positions according to genome build 37: cg05939324, chr12:7657479 (promoter); cg07264679, chr12:7657396 (promoter); cg03621974, chr12:7650334 (intron 4).

Table 5: Associations between *CD163* gene SNPs, visceral adipose tissue gene expression and methylation levels.

[24] and with associations previously demonstrated for *CD163* levels with obesity, diabetes and homeostatic model of insulin resistance index [29,30,32,33]. This supports the hypothesis that differential expression of *CD163* in VAT, potentially reflecting differential macrophage infiltration and activation, may have a physiological impact or reflect dysfunctional adipose tissue in severely obese individuals predisposed to the development of obesity-related metabolic complications.

Based on differential *CD163* VAT expression between MetS+ and MetS- groups and the potential impact of genetic variations on gene expression levels, identification of *CD163* SNPs was conducted. Associations of *CD163* sequence-based tSNPs with CVD risk factors (BP, glucose and lipid levels) were tested. Significant associations between *CD163* gene SNPs, plasma total-C and TG were observed. While a SNP located upstream the *CD163* gene was previously associated with creatine kinase levels but not with other hematological and biochemical traits in Japanese individuals, we report here the first association of *CD163* SNPs with lipid profile. Previous studies reported associations between sCD163 levels, TG and HDL-C levels [33] as well as a strong association of sCD163 with risk of developing T2DM [32]. Results presented here also support an association of rs11054195 with DBP, a trend toward a lower DBP being identified in rare allele carriers in comparison to homozygotes for the common allele. A positive association being reported between sCD163 and increased DBP [39], our results suggest a modulatory effect of *CD163* SNPs on this association requesting further examination in other studies. Associations reported between sCD163 and CVD risk factors thus reinforce potential modulatory effects of *CD163* gene variations reported here. In contrast, results presented here do not support an impact of *CD163* gene variations on fasting glucose or LDL-C levels while associations between sCD163, diabetes, insulin resistance and LDL-C have previously been reported [29,32,39].

The presence of SNPs in transcription factor (TF) binding sites and CpG sites methylation levels are known mechanisms for the modulation of gene expression levels [40,41]. The *CD163* promoter region was reported to contain putative binding sites for TF known to play a role in myeloid-specific gene expression and differentiation, as well as potential glucocorticoid receptor binding sites [42] which might contribute to the regulation of *CD163* gene expression following glucocorticoid treatment reported in monocytes and macrophages [43]. Further analyses were thus conducted on two CVD risk factor-associated SNPs (rs4883263, rs7980201) to explore potential mechanisms for differences in expression levels observed in VAT from our original subset of 14 individuals. While the rs7980201 SNP was associated with gene methylation levels of one of the promoter CpG site, none of the CVD risk factor-associated SNPs demonstrate association with gene expression levels. In addition, no association was observed between any of the 3 CpG site methylation levels and gene expression levels, thus suggesting that CVD risk factor-associated SNPs might exert their impact through other mechanisms. Accordingly, the amino acid change resulting SNP rs4883263 (c.1024G>A, p.Val342Ile) located in exon 5 and coding for the third *CD163* scavenger receptor cysteine-rich domain [44] was predicted to be damaging according to the SIFT program while PolyPhen-2 predicted this amino acid change to be benign. The rs4883263 SNP was also predicted to alter the binding of serine-arginine rich proteins SRSF1, 2 and 5 using the ESE finder 3.0 program (data not shown). Similarly, synonymous SNP rs7980201 (c.1515G>A), located in exon 7 was predicted to slightly increase the strength of the binding of SRSFR1 splicing protein (3.42 to 3.87). Collectively, these results suggest a possible impact of CVD risk factor-associated SNPs through altered protein or alternative splicing resulting in different isoforms [17,45]. However, plasma *CD163* protein levels were not available in the current study thus highlighting the need for additional studies into independent cohorts to further analyze the

relationship between *CD163* SNPs, gene methylation, expression and plasma *CD163* protein levels.

The present study identified a difference in VAT *CD163* gene expression level between MetS+ and MetS- individuals. Having the potential to induce the secretion of both pro-inflammatory (IL-1 β , TNF- α) and anti-inflammatory cytokines (NO and IL-6) [18], and being induced by IL-10 [46], *CD163* is positioned as a two-faced inflammatory modulator. A difference in *CD163* expression level or functionality thus has the potential to favor a switch from an inflammatory to an anti-inflammatory profile. For example, a recent study reported an increase in anti-inflammatory markers (*CD163* and IL-10) accompanied with a reduction in pro-inflammatory TNF- α in subcutaneous adipose tissue from obese compared to lean individuals. These results suggested a switch in macrophage polarization toward an anti-inflammatory profile with increasing obesity [30]. We speculate that increased *CD163* expression levels in MetS+ vs. MetS- individuals may reflect different macrophage infiltration in VAT or more extensive adipose tissue dysfunction in MetS+ individuals, both being associated with obesity-related complications [8,47]. The presence of the missense rs4883263 SNP may potentially alter *CD163* protein structure or functionality thus having an impact on the balance of pro-inflammatory and anti-inflammatory signals. The rs7980201 SNP being associated with a lower level of methylation for one of the promoter CpG site analyzed, and an inverse correlation being generally identified between promoter CpG site methylation and gene expression level, the present result suggests a possible impact of this SNP on inflammatory processes through a modulation of gene methylation levels which may reflect on gene expression levels. However, explorative analysis conducted in the present study did not reveal association between genotypes and gene expression levels or a correlation between methylation and expression levels.

The current study reports overexpression of *CD163* gene expression in VAT of severely obese men with vs. without the MetS. The *CD163* protein being exclusively expressed in monocytes/macrophages [17] and a difference in expression being observed between the MetS+ and MetS- groups, a difference in macrophage infiltration cannot be excluded. While our results demonstrate association of *CD163* SNPs with CVD risk factors, analyses conducted on the link between the presence of SNPs, gene methylation and expression levels suggest an influence of the rs7980201 SNP on CpG site methylation level but does not extend its impact to the gene expression level. Considering the presence of potential TF binding sites including glucocorticoid receptor binding sites in the *CD163* promoter region [42] and the relatively small number of selected individuals from our larger cohort (N=14), additional studies in larger cohorts focusing on the impact of SNPs on global *CD163* gene methylation and expression levels related to monocyte *CD163* mRNA and protein expression as well as its soluble form will be necessary to provide further insights into the biology of the associations identified here.

Conclusion

Assessing associations between SNPs within the *CD163* gene region and CVD risk factors based on differential expression of this gene in VAT from severely obese individuals with and without the MetS, the current study identified a potential contribution of *CD163* gene variations in inter-individual variability observed in obesity-related metabolic complications in severely obese individuals. Analysis conducted here on the impact of CVD risk factor-associated SNPs did not reveal a mechanistic contribution of the presence of *CD163* gene variations on gene methylation and expression levels. Further analyses

on the repercussion of *CD163* SNPs on monocytic/macrophage expression and s*CD163* are thus warranted, potentially providing mechanisms for associations identified here and the development of obesity-related metabolic complications.

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