Association between OLR1 K167N SNP and Intima Media Thickness of the Common Carotid Artery in the General Population

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Abstract

**Background and Purpose:** The lectin-like oxidised LDL receptor-1 (OLR1) gene encodes a scavenger receptor implicated in the pathogenesis of atherosclerosis. Although functional roles have been suggested for two variants, epidemiological studies on OLR1 have been inconsistent. **Methods** - We tested the association between the non-synonymous substitution K167N (rs11053646) and intima media thickness of the common carotid artery (CCA-IMT) in 2,141 samples from the Progression of Lesions in the Intima of the Carotid (PLIC) study (a prospective population-based study).

**Results:** Significantly increased IMT was observed in male carriers of the minor C (N) allele compared to GC and GG (KN and KK) genotype. Functional analysis on macrophages suggested a decreased association to Ox-LDL in NN carriers compared to KN and KK carriers which is also associated with a reduced OLR1 mRNA expression. Macrophages from NN carriers present also a specific inflammatory gene expression pattern compared to cells from KN and KK carriers.

**Conclusions:** These data suggest that the 167N variant of LOX-1 receptor affects the atherogenic process in the carotid artery prior to evidence of disease through an inflammatory process.

Introduction

Atherosclerosis is a complex disease. Endothelial dysfunction, activation and inflammation, proteolysis, apoptosis, platelet aggregation, thrombosis and angiogenesis are the processes involved in the phases of the disease [1]. The effects of environment on these phenotypes are also impacted by underlying genetic predisposition that may not impact all endo-phenotypes in the same way. The internalization of Ox-LDL has a critical effect in both endothelial dysfunction and inflammation [2]. This process is mediated by several scavenger receptors [3], including the lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) [4]. LOX-1 is mainly expressed in macrophages, endothelial, and smooth muscle cells. Its expression is induced by pro-inflammatory stimuli, such as shear stress, TNFα, LPS and infections [5,6,7]. LOX-1 is encoded by the OLR1 gene, mapped to chromosome 12p13 [8]. A Single Nucleotide Polymorphisms (SNP) on exon 4, rs11053646 (G501C), leads to an amino acidic substitution (lysine to asparagine at position 167, K167N). Functional analyses suggested that a change on the positive isopotential surface determined by this variant could lead to a decreased binding and internalization of Ox-LDL [9].

Despite the evidence for a functional role of this polymorphism, results from epidemiological studies are equivocal [10,11,12,13,14,15]. Of note, a gender specific association has been recently described between the C [N] allele and prevalence of carotid plaque in females of Dominican-Hispanic origin [16].

A direct association between circulating Ox-LDL and Intima Media Thickness (IMT) of the Common Carotid Artery has been demonstrated in previous studies [17,18,19]. Circulating Ox-LDL and IMT resulted inversely related to anti-OxLDL antibodies titulation, suggesting that immune response to Ox-LDL could have a protective role in the early phases of the disease [17,18,19].

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Since the N allele of rs11053646 is associated to lower levels of Ox-LDL internalization [9], we tested whether this allele is associated to CCA-IMT in the Progression of Lesions in the Intima of the Carotid Artery (PLIC) study (a prospective population-based study representative of the population of Northern Milan, Italy).

In addition, we investigated functional effects in macrophages obtained from carriers of different genotypes to test the hypothesis that N allele could have a reduced receptor activity. If this is true, that should result in a less effective Ox-LDL binding and internalization and therefore display lower levels of OLR1 RNA expression (as it is stimulated by Ox-LDL internalization itself).

**Methods**

**Study sample**

The use of human material in this study conforms to the principles outlined in the declaration of Helsinki. A cohort of 2,141 subjects attending the Atherosclerosis Centre in Bassini Hospital, Department of Pharmacological Sciences (University of Milan, Italy), was recruited for the PLIC study. This study has been previously widely described and the samples utilized in genetic studies [20,21,22,23,24,25,26].

**Genotyping**

Genomic DNA was extracted using the Flexigene DNA kit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. SNP genotyping was performed through the Taqman Genotyping Assay (ID: C__22273024__10, Applied Biosystems, Foster City, CA) on a BioRad machine. One μL (10–200 ng) of DNA was analysed for genotyping.

A Genotype confirmation of ~50 samples was obtained through Sanger sequencing on an ABI3130 machine (Methods S1).

**Statistical analysis**

Group differences were determined by using Analysis of Variance (ANOVA) for continuous variables and chi-square analysis for categorical variables. Group differences with P<0.1 were considered as suggestive and P<0.05 was deemed as statistically significant. Plots were generated using Excel and data were analyzed using the SPSS Software (http://www.spss.it/) on a Windows Machine.

**Peripheral blood mononuclear cell (PBMC) isolation and culture**

Peripheral blood mononuclear cells (PBMCs) were obtained from two subjects carrying the CC genotype (NN) and eight CG subjects (KN and KK). These subjects were healthy and of comparable age, gender and blood lipid profiles. Blood samples diluted 1:3 in phosphate-buffered saline (PBS; 15 mL, PH 7.4) were layered onto 4 mL of Ficoll-Hypaque (Amersham, Milan, Italy) and centrifuged at 300 g for 35 min. PBMCs were removed from the interface and washed twice (10 min, 300 g) in PBS before being counted.

PBMCs were re-suspended in RPMI supplemented with antibiotics and 10% serum bovine serum albumin and plated in 6-well plates and incubated for 1 1/2 hours at 37°C. Non-adherent cells were removed by rinses of PBS (4×). For addressing the association of Ox-LDL with macrophages, 1 week after the isolation, monocyte-derived macrophages were incubated with or without TNFα (10 ng/μL) for 18 h followed by Ox-LDL (6.25 or 12.5 μg/mL) for 1 h and then processed for FACS analysis.

**Expression analysis**

Total RNA was extracted from circulating monocytes and from monocytes-derived macrophages cells according to manufacturer’s Trizol protocol [27] and reverse transcription was performed as described [20]. Three μL of cDNA was amplified by real-time quantitative polymerase chain reaction (PCR) with 1 × Sybr green universal PCR mastermix (Applied Biosystems, Foster City, CA). The specificity of the Sybr green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. The melting peaks of the amplicons were as expected (data not shown). The primers used are reported in (Table S1). HPTR1 was used as internal reference. Each sample was analyzed in duplicate using the Applies Biosystems 7000 machine and each experiment was replicated twice. The PCR amplification was related to a standard curve ranging from 10⁻¹³ to 10⁻¹⁴ mol/L.

**Flow cytometry**

**Isolation and modification of low density lipoproteins.** LDLs (d = 1.019–1.063 g/mL) were isolated from fresh plasma of normolipidemic healthy volunteers by sequential ultracentrifugation [28]. Protein content was determined by the method of Lowry, using BSA as a standard [29]. Ox-LDL were generated with CaSO₄ 5 μM as described [30].

**Fluorescent labeling of lipoproteins.** For lipid labeling, Ox-LDL were incubated with the fluorescent dye DiO (300 μg DiO/mg OxLDL protein) in PBS for 18 h at 4 °C, passed over a PD10 column to remove unbound DiO, then centrifuged in a TL100 centrifuge at d = 1,063 g/mL for 2 h at 4 °C. DiO-labeled lipoproteins were passed through a PD10 column and protein content was determined by the method of Lowry [29].

**Cell-association studies.** Cells were incubated at 37°C for 1 h with the indicated concentrations of Ox-LDL labeled with DiO. Cells were then washed three times with cold PBS, detached by scraping, fixed in 1% paraformaldehyde and immediately subjected to fluorescence flow cytometry using a FACSscan (Becton Dickinson). For each sample 10,000 events were analyzed; data were processed using the CellQuest program (Becton Dickinson) [31].

**Results**

The relative frequencies of the three OLR1 genotypes are shown in Table 1. No deviation from Hardy Weinberg equilibrium was observed.

![Image](Image 58x24 to 76x41)

The presence of the N allele was not associated with any of the cardiometabolic variables analysed (Table 2) and the increase in IMT observed was not statistically significant (0.64±0.07 for KK, KN and NN respectively, p = n.s.). As a recent paper showed a gender related effect of the N allele, we decided to stratify the results for gender (Table 3). The N allele was associated to a higher IMT in men. In women N allele was not associated with any of the variables. The increase in IMT observed was not statistically significant (0.66±0.11 mm and 0.69±0.07 for KK, KN and NN respectively).

Table 1. Observed and expected frequencies of OLR1 K167N polymorphism in the PLIC population and comparison with those reported in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs = 11053646).

|          | KK   | KN   | NN   | p-value |
|----------|------|------|------|---------|
| Observed | 1988 (87%) | 283 (12%) | 5 (0.02%) | 0.12    |
| Expected | 1992.4 (88%) | 274.1 (12%) | 9.4 (0.04%) |
| dbSNP frequency CEU | 75% | 25% | 0% |

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of disease, we confirmed in vivo that NN macrophages internalize Ox-LDL to a lower extent and found evidences suggestive for an immune reaction to circulating Ox-LDL in early phases of the disease.

**Discussion**

The main finding of this paper is that males from the general population with the C (N) allele of the OLR1 gene have an increased IMT. Since no other phenotype resulted associated to this variant, this suggests it to be an independent association. To date, conclusive results on the epidemiology of OLR1 gene polymorphisms were obtained. Both a protective role for MI and CAD severity [10,12,14] and a risk role for MI and hypertension [11,15] for the C (N) allele were proposed. Also, the replication of these results was rarely tested and never obtained [14], leading to the conclusion that these were spurious associations. Furthermore, a recent meta-analysis considered all studies that have been published on the relationship between OLR1 polymorphisms and MI, finding partially contrasting results and an overall effect that is non-significant when considering the K167N variation [33].

Recently, a study reported the association between the C (N) allele and carotid plaque prevalence in 167 women from a Dominican-Hispanic population [16]. We extend these findings, showing an allele’s association in 926 males from the PLIC population and analysing the molecular players and pathological responses associated with leucocytes and macrophages from NN, KN and KK subjects.

Table 3. Statistical observations on the association between OLR1 polymorphisms in males.

|                | K167N (rs11053646) | p-value |
|----------------|-------------------|---------|
|                | KK                | KNaNN   |
| Systolic blood | 135.83+/−0.630    | 133.39+/−1.47 | ns   |
| pressure (mmHg)|                  |         |
| Diastolic blood| 84.42+/−0.34      | 83.17+/−0.91 | ns   |
| pressure (mmHg)|                  |         |
| Total cholesterol (mmol L/) | 221.77+/−0.890 | 220.07+/−2.400 | ns |
| HDL cholesterol (mmol L/) | 56.13+/−0.340 | 54.07+/−0.810 | ns   |
| LDL cholesterol (mmol L/) | 144.205+/−0.820 | 144.717+/−2.180 | ns   |
| Triglycerides (mmol L/) | 108.11+/−1.420 | 105.58+/−3.280 | ns   |
| IMT (mm) | 0.663+/−0.004 | 0.694+/−0.011 | 0.050* |

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Table 2. K167N polymorphism in the PLIC population.

|                | K167N (rs11053646) | p-value |
|----------------|-------------------|---------|
| Age (years)    | 54.33+/−0.260     | 54.43+/−0.680 | ns |
| Systolic blood  | 133.33+/−0.420    | 131.23+/−1.050 | 0.070 |
| pressure (mmHg)|                  |         |
| Diastolic blood | 82.96+/−0.220     | 81.85+/−0.600 | 0.095 |
| pressure (mmHg)|                  |         |
| Total cholesterol (mmol L/) | 221.77+/−0.890 | 220.07+/−2.400 | ns |
| HDL cholesterol (mmol L/) | 56.13+/−0.340 | 54.07+/−0.810 | ns   |
| LDL cholesterol (mmol L/) | 144.205+/−0.820 | 144.717+/−2.180 | ns   |
| Triglycerides (mmol L/) | 108.11+/−1.420 | 105.58+/−3.280 | ns   |
| IMT (mm) | 0.645+/−0.003 | 0.658+/−0.007 | 0.080 |

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population. It is known, in fact, that Hispanics have different susceptibility to cardiovascular disease, only partially explainable by environmental factors [34]. Furthermore OLR1 variants display highly different frequencies in different populations (p = 0.0001 comparing European ancestry HapMap populations and African HapMap populations, and p = 0.04 comparing European ancestry populations and Mexicans in HapMap populations. Details about analyses on HapMap samples are available upon request). Second, gene-regulation for many quantitative traits differs significantly between males and females. In particular, evidences suggested a differential genotype by sex interaction on variation of Paraoxonase-1 (PON1, a calcium dependent esterase known to have antioxidant properties) activity in Mexican American populations [35]. We tested for interaction between available functional variants on both OLR1 and PON1 from the HapMap dataset and found suggestive evidences for differences in gene-by-gender

**Figure 1.** Gene expression levels from Peripheral Mononuclear Cells (PBMCs) of KK, KN and NN carriers. Expression levels are measured by the log (ΔΔCt) obtained comparing each gene’s expression with that of the housekeeping gene, HPRT1. (*Nf-kB*: nuclear factor kappa-light-chain-enhancer of activated B cells, *ERK1/2*: extracellular related kinase 1/2, *IL-6*: Interleukin-6, *CD40*: cluster of designation 40, *CX3CR1*: CX3 chemokine receptor 1, *TLR-4*: Toll-like receptor 4, *MMP*: metalloproteinase). doi:10.1371/journal.pone.0031086.g001

**Figure 2.** OLR1 expression in KK, KN and NN in differentiated macrophages. (*Nf-kB*: nuclear factor kappa-light-chain-enhancer of activated B cells, *ERK1/2*: extracellular related kinase 1) (* p < 0.05 vs KK). doi:10.1371/journal.pone.0031086.g002
interactions in populations with different ancestry (details about analyses on HapMap samples are available upon request). Third, Wang and co-workers focused their analysis on the presence of plaque, (advanced atherosclerosis) while we focused on IMT (atherogenesis) thus suggesting a dual role for OLR1 in different phases of the atherogenic process.

Although we have to recognize some limits of our study, including the low number of NN individuals, this is the first study which was able to directly carry on functional tests on human NN PBMCs and macrophages and represents, to date, the second largest study which investigated the role of LOX-1 functional polymorphism in the onset of cardiovascular disorders. Therefore, these findings support the relevance of OLR1 in vascular disorders at epidemiological and functional level.

Supporting Information

Methods S1 Sequencing of samples: All of the CC (NN) and 50 of the CG (KN) and GG (KK) genotypes were verified through sequencing using forward primer: ATGCACGTGAGAGAACTAAGGG and reverse primer: TGGCTCTCAAACAAGAATTCC (Applied Biosystems, Foster City, CA). Two CC individuals turned out to be CG, but since for Statistical Analyses KK and KN were considered as a single group, results were not affected. All GG and CG individuals were confirmed.

Table S1 Primer sequences for Gene Expression Analysis ((Nf-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, ERK1/2: extracellular related kinase 1/2, IL-6: Interleukin-6, CD40: cluster of designation 40, CX3CR1: CX3 chemokine receptor 1, TLR-4: Toll-like receptor 4, MMP: metalloproteinase).

Table S2 Gene expression levels in PBMCs obtained from KK and NN. (Nf-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, ERK1/2: extracellular related kinase 1/2, IL-6: Interleukin-6, CD40: cluster of designation 40, CX3CR1: CX3 chemokine receptor 1, TLR-4: Toll-like receptor 4, MMP: metalloproteinase).

Table S3 OLR1, NF-kB and ERK1/2 expression levels in differentiated macrophages obtained from KK and NN PBMCs. (Nf-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, ERK1/2: extracellular related kinase 1/2).

Table S4 OxLDL association levels to macrophages obtained from the different genotypes.

Author Contributions

Conceived and designed the experiments: IMP GDN GN ALC. Performed the experiments: IMP GDN LV KG FA LG ST LC AP. Analyzed the data: IMP GDN GA. Contributed reagents/materials/analysis tools: IMP GDN FA GN ALC. Wrote the paper: IMP GDN LV KG FA LG LC AP FR GN ALC.

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