Title

Common variants in OSMR contribute to carotid plaque vulnerability

Short Title

Common variants in OSMR and plaque vulnerability

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MANUSCRIPT CONTENTS
Manuscript of 5,854 words, 3 tables, 2 figures and Supplemental Material.

JOURNAL SUBJECT TERMS
Epigenetics, Genetics, Atherosclerosis, Cardiovascular disease
Abstract

Background

Oncostatin M (OSM) signaling is implicated in atherosclerosis, however the mechanism remains unclear. We investigated the impact of common genetic variants in OSM and its receptors, OSMR and LIFR, on overall plaque vulnerability (based on macrophage, collagen, smooth muscle cell and fat content) and on seven individual atherosclerotic plaque phenotypes (calcification, collagen, atheroma size, macrophages, smooth muscle cells, vessel density and intraplaque hemorrhage).

Methods and results

We queried Genotype-Tissue Expression (GTEx) data and selected one variant, rs13168867 (C allele), that associated with decreased OSMR expression and one variant, rs10491509 (A allele), that associated with increased LIFR expression in arterial tissue. No variant was associated to significantly altered OSM expression.

We associated these two variants with plaque characteristics from 1,443 genotyped carotid endarterectomy patients in the Athero-Express Biobank Study. The rs13168867 variant in OSMR was significantly associated with an increased overall plaque vulnerability ($\beta = 0.118 \pm \text{s.e.} = 0.040$, $p = 3.00\times10^{-3}$, C allele). With respect to different plaque phenotypes, this variant showed strongest associations with intraplaque fat ($\beta = 0.248 \pm \text{s.e.} = 0.088$, $p = 4.66\times10^{-3}$, C allele) and collagen content ($\beta = -0.259 \pm \text{s.e.} = 0.095$, $p = 6.22\times10^{-3}$, C allele). No associations were found for rs10491509 in the LIFR locus.

Conclusion

Our study suggests that genetically decreased arterial OSMR expression, possibly resulting in decreased OSM signaling, contributes to increased carotid plaque vulnerability.

Keywords: Atherosclerosis, plaque, OSM, OSMR
1. Introduction

Oncostatin M (OSM) is an inflammatory cytokine that is released by activated monocytes, macrophages, T-lymphocytes and neutrophils, and mediates its effects through binding to either the glycoprotein (gp) 130/oncostatin M receptor (OSMR) heterodimer or the gp130/leukemia inhibitory factor receptor (LIFR) heterodimer. Binding of OSM to either of the receptor heterodimers can activate multiple pathways, including the janus kinase (JAK)/signal transduction and activator of transcription (STAT), the mitogen-activated protein kinase (MAPK), and the Phosphoinositide 3-kinase (PI3K)/AKT pathway. It is suggested that the ratio of the two receptor types expressed on the cell membrane is a potential regulatory mechanism for the multiple and sometimes opposing effects that are exerted by OSM. The cytokine is associated to multiple inflammatory diseases, including chronic periodontitis, rheumatoid arthritis and inflammatory bowel disease.

There are multiple indications, that OSM is involved in atherosclerosis. OSM is present in both murine and human atherosclerotic plaques and OSMR−/−-ApoE−/− mice show reduced plaque size and improved plaque stability compared to their OSMR expressing littermates, indicating that OSM drives atherosclerosis development. To our knowledge, no studies have been performed to investigate the involvement of LIFR in OSM driven atherosclerosis development. However, we previously showed that OSM signals through both receptors simultaneously to induce activation in human endothelial cells, suggesting that also LIFR is involved in atherosclerosis development.

Little is known about the effect of OSM on plaque composition. Since OSM affects multiple cell types and processes, it is difficult to predict how OSM contributes to atherosclerotic plaque formation. As OSM promotes angiogenesis, endothelial activation, vessel permeability and osteoblastic differentiation, it hypothetically results in a higher intraplaque microvessel density and intraplaque hemorrhages and plaque calcification, thereby contributing to the formation of a vulnerable plaque. On the other hand, OSM also promotes fibroblast proliferation, collagen formation, smooth muscle cell...
proliferation\textsuperscript{12} and M2 macrophage polarization\textsuperscript{21}, hypothetically resulting in enhanced fibrosis and attenuates inflammation, thereby contributing to plaque stabilization\textsuperscript{22-24}.

We aimed to investigate these theorized opposing effects of OSM signaling on the atherosclerotic plaque using data from the Athero-Express Biobank Study, which comprises a large collection of human plaque specimens obtained through carotid endarterectomy\textsuperscript{25}. Common genetic variation in gene expression is key to disease susceptibility, and \textit{cis}-acting genetic variants, single-nucleotide polymorphisms (SNPs), have been mapped to expression quantitative trait loci (eQTLs)\textsuperscript{26}. Likewise, eQTLs modulate transcriptional regulation of \textit{OSM}, \textit{OSMR}, and \textit{LIFR} in arterial tissues. We hypothesized that eQTLs for these genes, can be used as proxies of gene expression to examine the effect on overall plaque vulnerability\textsuperscript{27} and individual plaque characteristics, including collagen, lipid, macrophage and smooth muscle cell content, calcification, and intraplaque microvessel density and hemorrhage.

2. Materials and methods

2.1 Sample collection

The Athero-Express Biobank Study (https://www.atheroexpress.nl) contains plaque material of patients that underwent carotid endarterectomy (CEA) or femoral endarterectomy at two Dutch tertiary referral centers\textsuperscript{25}. Details of the study design were described before. Briefly, blood and plaque material were obtained during endarterectomy and stored at \(-80\)\textdegree C. Only CEA patients were included in the present study. All patients provided informed consent and the study was approved by the medical ethics committee.

2.2 Athero-Express genotyping, quality control, and imputation

Details of genotyping have been previously described\textsuperscript{28}. In short, DNA was extracted from EDTA blood or (when no blood was available) plaque samples of 1,858 consecutive patients from the Athero-Express Biobank Study and genotyped in 2 batches. For the Athero-Express Genomics Study 1 (AEGS1) 836 patients, included between 2002 and 2007, were
genotyped using the Affymetrix Genome-Wide Human SNP Array 5.0 (SNP5) chip (Affymetrix Inc., Santa Clara, CA, USA). For the Athero-Express Genomics Study 2 (AEGS2) 1,022 patients, included between 2002 and 2013, were genotyped using the Affymetrix Axiom® GW CEU 1 Array (AxM). Both studies were carried out according to OECD standards. After genotype calling, we adhered to community standard quality control and assurance (QCA) procedures of the genotype data from AEGS1 and AEGS2. Samples with low average genotype calling and sex discrepancies (compared to the clinical data available) were excluded. The data was further filtered on 1) individual (sample) call rate > 97%, 2) SNP call rate > 97%, 3) minor allele frequencies (MAF) > 3%, 4) average heterozygosity rate ± 3.0 s.d., 5) relatedness (pi-hat > 0.20), 6) Hardy-Weinberg Equilibrium (HWE p < 1.0×10^{-6}), and 7) population stratification (based on HapMap 2, release 22, b36) by excluding samples deviating more than 6 standard deviations from the average in 5 iterations during principal component analysis and by visual inspection as previously described. After QCA 657 samples and 403,789 SNPs in AEGS1, and 869 samples and 535,983 SNPs in AEGS2 remained. Before phasing using SHAPEIT2, data was lifted to genome build b37 using the liftOver tool from UCSC (https://genome.ucsc.edu/cgi-bin/hgLiftOver). Finally, data was imputed with 1000G phase 3 and GoNL 5 as a reference.

2.3 Variant selection

We queried data from the Genotype-Tissue Expression (GTEx) Portal (https://gtexportal.org) for variants that alter OSM expression in the blood, and OSMR or LIFR expression in arterial tissue. We selected common variants with a MAF >3%, which yielded 2 variants in total. We harmonized the effect alleles and effect sizes from these eQTLs to the Athero-Express Biobank Study data.

2.4 Plaque phenotyping

The (immuno)histochemical analyses of plaque phenotypes have been described previously. Briefly, the culprit lesion was identified directly after dissection, fixed in
4% formaldehyde and embedded in paraffin. The tissue was cut in 5µm sections on a cryotome for (immuno)histochemical analysis by pathology experts. Calcification (hematoxylin & eosin, H&E) and collagen content (picrosirius red) were semi-quantitatively scored and defined as no/minor or moderate/heavy. Atheroma size (H&E and picrosirius red) was defined as <10% or ≥10% fat content. The amount of macrophages (CD68) and smooth muscle cells (ACTA2) were quantitatively scored and classified as percentage of plaque area. The presence of intraplaque hemorrhage (H&E and fibrin) was defined as absent or present, and vessel density was classified as the number of intraplaque vessels (CD34)/ hotspot.

2.5 Plaque vulnerability

Assessment of overall plaque vulnerability was performed as previously described\(^2^7\). In short, the amount of macrophages and smooth muscle cells were also semi-quantitatively defined as no/minor or moderate/heavy. Each plaque characteristic that defines a stable plaque (i.e. no/minor macrophages, moderate/heavy collagen, moderate/heavy smooth muscle cells and <10% fat) was given a score of 0, while each plaque characteristic that defines a vulnerable plaque (i.e. moderate/heavy macrophages, no/minor collagen, no/minor smooth muscle cells and ≥10% fat) was given a score of 1. The score of each plaque characteristic was summed resulting in a final plaque score ranging from 0 (most stable plaque) to 4 (most vulnerable plaque). Intraobserver and interobserver variability were examined previously and showed good concordance (κ 0.6-0.9)\(^3^3\).

2.6 Statistical analyses

Quantitatively scored characteristics (macrophages, smooth muscle cells, and the vessel density) were Box-Cox transformed\(^3^4\) to obtain a normal distribution. Association of the common variants with continuous parameters were statistically tested with linear regression and the categorical parameters with logistic regression. Data was corrected for age, sex, genotyping chip, and genetic ancestry using principal components 1 through 4. Correction for multiple testing resulted in a corrected p-value of p = 0.05/(7 plaque
phenotypes + plaque vulnerability) \times 2 \text{ common variants} = 3.13 \times 10^{-3}. \text{ The power of the study was estimated at } \pm 75\% \text{ based on a sample size of 1,443, a minor allele frequency (MAF) of 0.409 and a relative risk of 1.28} (\text{http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/}, \text{ Supplemental Figure 1}).

3. Results

3.1 Baseline characteristics

A total of 1,443 patients that underwent carotid endarterectomy were genotyped and included in this study. The genotyped groups (AEGS1 and AEGS2) are not overlapping. As we previously showed that the baseline characteristics of both groups are comparable\textsuperscript{28}, the groups were combined for overall plaque vulnerability and phenotype analyses. Baseline characteristics of the combined groups are shown in Table 1.

3.2 Common variants altering OSM, OSMR and LIFR expression

OSM is secreted by neutrophils\textsuperscript{4}, monocytes\textsuperscript{2}, macrophages\textsuperscript{2} and T-cells\textsuperscript{3}, and acts through binding to OSMR and LIFR\textsuperscript{5–7} in the arterial wall\textsuperscript{13,35}. Thus we queried data from the Genotype-Tissue Expression project (GTEx)\textsuperscript{26} for SNPs that alter OSM expression in whole blood and LIFR and OSMR expression in arterial tissue. There were no significant eQTLs for OSM, but there were two eQTLs that associated with either altered OSMR (rs13168867) or LIFR (rs1049150) expression in arterial tissue. The C allele of rs13168867 showed the strongest association with decreased OSMR expression in the tibial artery (Figure 1A), and the A allele of rs1049150 showed the strongest association with increased LIFR expression in the aortic artery (Figure 1B). Cross-tissue meta-analysis showed that these variants have > 0.9 m-values in both tibial and aortic artery tissue, indicating a high probability that they are single cis-eQTLs in both tissues (Supplemental Figure 2 and 3).

3.3 Genetic association with plaque vulnerability

To determine the effect of OSM signaling on the overall plaque vulnerability, we correlated the rs13168867 and rs1049150 genotypes to the overall plaque vulnerability, which was
given a score ranging from 0 (least vulnerable plaque) to 4 (most vulnerable plaque). The effect allele of variant rs13168867 in the OSMR locus was significantly correlated with an increased overall plaque vulnerability ($\beta = 0.118 \pm s.e. = 0.040$ (C allele), $p = 3.00 \times 10^{-3}$, Table 2), which is visualized in Figure 2. No association was observed with rs10491509 and overall plaque vulnerability.

3.4 Genetic association with plaque phenotypes

To determine the effect of OSM signaling on the plaque phenotype, we assessed the association between rs13168867 and rs10491509 and seven plaque phenotypes in the Athero-Express Biobank Study. The strongest associations were observed between the effect allele of variant rs13168867 in the OSMR locus and intraplaque fat ($\beta = 0.248 \pm s.e. = 0.088$ (C allele), $p = 4.66 \times 10^{-3}$), and collagen content ($\beta = -0.259 \pm s.e. = 0.095$ (C allele), $p = 6.22 \times 10^{-3}$, Table 3). No associations were observed between rs10491509 and any of the plaque phenotypes.

4. Discussion

We investigated whether common variants associated to arterial gene expression, eQTLs, near OSM, OSMR and LIFR affect overall plaque vulnerability and phenotype. We showed that one cis-acting eQTL (rs13168867), associated with reduced OSMR expression arterial tissue, is associated with increased plaque vulnerability. This suggests that a decrease in OSMR expression and therefore possibly a decrease in OSM signaling, increases the chance on a vulnerable plaque. To gain further insight into the role of genetically decreased OSMR expression on plaque vulnerability, we examined the effect of rs13168867 on individual plaque characteristics in more detail. The strongest associations were found for rs13168867 with increased intraplaque fat and decreased collagen content, suggesting that reduced OSM signaling results in a larger lipid core and less fibrosis - in line with a more vulnerable plaque phenotype. None of the other plaque characteristics were associated with rs13168867.
The increase in intraplaque fat content that is associated with genetically decreased arterial OSMR expression can be related to the effect of OSM signaling on endothelial cells. OSM enhances ICAM-1 expression, but not VCAM-1 expression on endothelial cells, suggesting that OSM enhances recruitment of the non-classical monocyte subset has a protective effect on the endothelium. Furthermore, this monocyte subset is biased to turn into the M2 macrophage subset, which is associated with plaque regression. OSM could even accelerate this process as OSM induces M2 macrophage polarization. Yet, this hypothesis remains to be investigated and future studies should explore whether genetically reduced OSM signaling indeed increases intraplaque lipid content by reduced recruitment of non-classical monocytes and impaired M2 macrophage polarization.

The decrease in collagen content associated to genetically decreased OSM signaling could be attributed to the increase in fibroblast proliferation and enhancement of collagen formation that is induced by OSM. Moreover, OSM enhances liver fibrosis in mice and is upregulated in patients with pulmonary fibrosis. Future studies are required to investigate if decreased OSM signaling indeed contributes to a decreased collagen content in plaques by reduced fibroblast proliferation and collagen formation.

No significant associations were found for rs10491509 in the LIFR locus and overall plaque vulnerability or any of the investigated plaque phenotypes. This suggests that an increase in LIFR expression does not affect OSM signaling or that OSMR is the dominant receptor in OSM signaling regarding the OSM related effects on atherosclerotic plaque vulnerability and phenotypes.

Recent developments in single-cell expression analyses might extend on the present study by investigating which cell types, that are present in the plaque, most abundantly express OSM, OSMR and LIFR. Furthermore, it would be interesting to investigate if the OSMR/LIFR expression ratio correlates with plaque vulnerability and if this ratio might be a predictor of plaque vulnerability.

Interestingly, a previous study showed that OSMR deficient mice have more stable plaques, which runs counter to our findings. This controversy could be explained by the
differences in human and murine OSM signaling. In humans OSM binds to OSMR and LIFR with the same affinity, while murine OSM has a much stronger affinity for OSMR. In point of fact, while homologous, the sequence similarity of human OSM, OSMR, LIFR with murine is moderate at best (64.75%, 70.79%, 78.51%, respectively based on data from GeneCards) and might directly impact receptor affinity.

Based on our data we can conclude that the variant rs13168867 in the OSMR locus is associated with increased plaque vulnerability. Given the multiple testing burden for individual plaque characteristics, it remains unclear through which precise biological mechanisms OSM signaling exerts its effects on plaque morphology, although our data point towards lipid metabolism and extracellular matrix remodeling. Compared to genome-wide association studies that include thousands of individuals, the Athero-Express Biobank Study is relatively small (n = 1,443), and given its design finite in size. However it is well suited to examine the effect of common disease associated genetic variation on plaque morphology and characteristics. Indeed, we estimated the power at ±75% given a MAF = 0.40 (approximately the frequency of rs13168867) and relative risk = 1.28.

5. Conclusion

We associated one eQTL in the OSMR locus, which associates with decreased arterial OSMR gene expression, with increased human plaque vulnerability in the Athero-Express Biobank Study. Further analyses of plaque phenotypes showed the strongest associations of this eQTL with increased intraplaque fat and decreased collagen content. No associations were found between an eQTL in the LIFR locus and either plaque vulnerability or any of the investigated plaque characteristics. In contrast to earlier mouse studies, our observations in human derived samples suggest that genetically decreased OSMR arterial expression contributes to increased carotid plaque vulnerability. Further, our study underscores the need to study prospective therapeutic targets derived in experimental models through human genetics.

Funding
SWvdL is funded through grants from the Netherlands CardioVascular Research Initiative of the Netherlands Heart Foundation (CVON 2011/B019 and CVON 2017-20: Generating the best evidence-based pharmaceutical targets for atherosclerosis [GENIUS I&II]). This work was supported by ERA-CVD, grant number: 01KL1802. FWA is supported by UCL Hospitals NIHR Biomedical Research Centre. DvK, HP and DT are funded through the FP7 EU project CarTarDis (FP7/2007-2013) under grant agreement 602936. HP received funding from the TNO research program “Preventive Health Technologies”.

Disclosures

DvK is employee of Quorics B.V. and DT is employee of SkylineDx B.V and Quorics B.V. Quorics B.V. and SkylineDx B.V. had no part whatsoever in the conception, design, or execution of this study nor the preparation and contents of this manuscript.

Acknowledgments

We would like to thank dr. Jessica van Setten and acknowledge her graciously for imputing our datasets using an in-house developed imputation pipeline. Evelyn Velema and Petra Homoet-Van der Kraak are graciously acknowledged for the immunohistochemical stainings. We also acknowledge the support from the Netherlands CardioVascular Research Initiative from the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development and the Royal Netherlands Academy of Sciences (“GENIUS I & II”, CVON2011-19) and the TNO research program “Preventive Health Technologies”.

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**Table 1. Baseline characteristics of genotyped CEA patients from the Athero-Express Biobank Study.** Cerebrovascular disease history is defined by ischemic stroke prior to surgery. Coronary artery disease history includes coronary artery disease, myocardial infarction, percutaneous coronary intervention, and coronary artery bypass graft. Peripheral disease history includes diagnosed peripheral arterial occlusive disease, femoral artery interventions, and ankle-brachial index <70. Type 2 diabetes mellitus includes all individuals with diagnosed type 2 diabetes mellitus and those on appropriate medication. Hypertension includes all individuals with self-reported hypertension. Current smokers include all individuals smoking up to 6 months until the surgery date. BMI, kg/m². eGFR rate was based on the Modification of Diet in Renal Disease formula, mL/min/1.73m². Anti-hypertensives include all anti-hypertension medication. Anti-thrombotics include clopidogrel, dipyridamole, acenocoumarin, ascal, and anti-platelet drugs. Missingness shows the percentage of the patients of which we lack information on the specific patient characteristic.

| Patient characteristics | AEGS 1 and 2 N = 1,443 | Missingness (%) |
|-------------------------|-------------------------|-----------------|
| **Sex, male, n (%)**    | 976 (64.0)              | 5.7             |
| **Age in years, mean (SD)** | 68.84 (9.33)          | 5.7             |
| **History**             |                         |                 |
| Cerebrovascular disease, n (%) | 478 (33.2)             | 5.7             |
| Coronary artery disease, n (%) | 430 (29.9)             | 5.8             |
| Peripheral artery disease, n (%) | 297 (20.7)             | 5.8             |
| **Risk factors**        |                         |                 |
| Type 2 diabetes mellitus, n (%) | 332 (23.1)             | 5.7             |
| Hypertension, n (%)     | 1017 (73.0)             | 8.7             |
| Current smoker, n (%) | 492 (34.9) | 7.5 |
|----------------------|------------|-----|
| BMI, median [IQR]    | 26.0 [24.0-28.4] | 11.5 |
| eGFR, median [IQR]   | 72.3 [58.7-85.4] | 8.1 |
| Total cholesterol in mmol/L, median [IQR] | 4.38 [3.60-5.25] | 22.8 |
| LDL in mmol/L, median [IQR] | 2.40 [1.81-3.13] | 27.8 |
| HDL in mmol/L, median [IQR] | 1.06 [0.87-1.30] | 25.0 |
| Triglycerides in mmol/L, median [IQR] | 1.50 [1.08-2.04] | 24.6 |
| **Medication**        |            |     |
| Antihypertensives, n (%) | 1110 (77.2) | 5.8 |
| Lipid lowering drugs, n (%) | 1112 (77.4) | 5.8 |
| Antithrombotics, n (%) | 1272 (88.6) | 6.0 |
| **Symptoms**          |            |     |
| Asymptomatics, n (%)  | 195 (13.6) | 6.0 |
| Ocular, n (%)         | 221 (15.4) | 6.0 |
| TIA, n (%)            | 634 (44.2) | 6.0 |
| Stroke, n (%)         | 384 (26.8) | 6.0 |
Table 2. *OSMR* and *LIFR* variants and their association with overall plaque vulnerability

For each variant, the association with overall plaque vulnerability is given. NEF: the normalized effect size on expression (from GTEx Portal, www.gtexportal.org)\(^{25}\); Alleles: the effect allele and the other allele, respectively; EAF: effect allele frequency; Info: estimated imputation score; \(\beta\): effect size; s.e.: standard error; \(P\): p-value of association.

| Gene | Variant | NEF | Alleles | EAF  | Info  | \(\beta\) (s.e.) | \(P\)     |
|------|---------|-----|---------|------|-------|-----------------|----------|
| *OSMR* | rs13168867 | -0.13 | C/T    | 0.393 | 0.999 | 0.118 (0.040) | 3.00 x 10\(^{-3}\) |
| *LIFR* | rs10491509 | 0.29  | A/G    | 0.351 | 0.989 | 0.001 (0.043) | 0.981    |
Table 3. OSMR and LIFR variants and their association with plaque phenotypes

For each variant, the association with plaque phenotypes is given. NEF: the normalized effect size on expression; Alleles: the effect allele and the other allele, respectively; EAF: effect allele frequency; Info: estimated imputation score; β: effect size; s.e.: standard error; P: p-value of association. Calcification and collagen were classified as no/minor vs moderate/heavy, fat content as 10% vs >10% fat of plaque area, intraplaque hemorrhage was classified as absent vs present. Smooth muscle cells and macrophages were classified as Box-Cox transformed percentage of plaque area and vessel density as Box-Cox transformed number of vessels/ hotspot.

| Gene | Variant | NEF | Alleles | EAF | Info | Phenotype                  | β (s.e.)         | P       |
|------|---------|-----|---------|-----|------|----------------------------|-----------------|---------|
| OSMR | rs13168867 | -0.13 | C/T    | 0.393 | 0.999 | Calcification               | 0.036 (0.077)  | 0.637   |
|      |          |      |         |      |      |                            |                 |         |
|      |          |      |         |      |      | Collagen                   | -0.259 (0.095) | 6.22 x 10^-3 |
|      |          |      |         |      |      | Fat content                | 0.248 (0.088)  | 4.66 x 10^-3 |
|      |          |      |         |      |      | Intraplaque hemorrhage     | -0.014 (0.080) | 0.862   |
|      |          |      |         |      |      | Smooth muscle cells        | 0.001 (0.011)  | 0.913   |
|      |          |      |         |      |      | Vessel density             | -0.000 (0.004) | 0.976   |
|      |          |      |         |      |      | Macrophages                | 0.004 (0.015)  | 0.809   |
| LIFR | rs10491509 | 0.29 | A/G    | 0.351 | 0.989 | Calcification               | 0.046 (0.082)  | 0.577   |
|                          |          |        |
|--------------------------|----------|--------|
| Collagen                 | 0.134    | 0.194  |
|                          | (0.104)  |        |
| Fat content              | 0.086    | 0.363  |
|                          | (0.094)  |        |
| Intraplaque hemorrhage   | 0.071    | 0.414  |
|                          | (0.086)  |        |
| Smooth muscle cells      | -0.003   | 0.840  |
|                          | (0.012)  |        |
| Vessel density           | 0.002    | 0.577  |
|                          | (0.004)  |        |
| Macrophages              | 0.015    | 0.354  |
|                          | (0.016)  |        |
Figure 1: Association of OSMR and LIFR variants in arterial tissues. Per variant, the normalized expression of OSMR (A) and LIFR (B) is given in arterial tissue. Data from GTEx Portal (www.gtexportal.org). NES = Normalized effect size. In aortic arterial tissue, rs13168867 had a NES of -0.123 and in tibial arterial tissue, rs10491509 had a NES of 0.0881 (Supplementary Figure 2 and 3).
Figure 2: Association of OSMR and LIFR variants with overall plaque vulnerability. The plaques were given a score ranging from 0 (least vulnerable plaque) to 4 (most vulnerable plaque) and divided in three groups (Group 1 contains plaque vulnerability scores 0 and 1, group 2 contains the plaque vulnerability score 2 and group 3 contains the plaque vulnerability scores 3 and 4). The bars represent the proportion of each group per genotype for rs13168867 in the OSMR locus (A) and rs10491509 in the LIFR locus (B). A bar chart showing the proportion of each individual score is shown in Supplemental Figure 4.