Gene expression differences between stroke-associated and asymptomatic carotid plaques

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Supplemental Methods

RNA extraction

RNA was obtained from a longitudinal slice of the CP containing part of the atheroma core. Total RNA was extracted using Trizol® reagent (Invitrogen Life Technologies) and purified with an RNeasy Total RNA Isolation Kit (Qiagen) according to the manufacturers’ recommendations. One CP from an asymptomatic patient originally included in the microarray group did not yield enough RNA for microarray analysis, but this sample was included in the qPCR analysis as part of the extended replication group.

RNA quality was assessed both using conventional spectrophotometry (concentration and A260/A280 ratio ≥2) and microcapillary electrophoresis (RNA 6000 Nano LabChip Kit, Agilent Bioanalyzer 2100). Some of the samples were moderately degraded but all still showed clear ribosomal 18S and 28S peaks, only moderately elevated baseline and the 28S:18S rRNA ratios over 1. Examples of representative electropherograms are shown in Supplemental figure 1.
**Microarray Analysis**

Original data files are available at ArrayExpress ID: E-MEXP-2257 (http://www.ebi.ac.uk/microarray-as/ae/).

The scanned microarray images and Affymetrix quality measures were checked using MASv5.1 software (Affymetrix). Signal intensities were calculated and hybridization data normalized across arrays using the Robust Multi-array Average method (RMA) [1]. Genes expressed at a reliable level and showing differential expression were identified by filtering: 1) probes with a raw signal <100 in >50% of the samples in both groups and 2) probes <1.5-fold difference in the mean signal between SCPs and ACPs. These criteria are based on hybridisation data on a technical replicate (the RNA sample from one plaque was hybridised to two arrays): when these signal and fold-change criteria were applied, 90% of the false positives could be excluded. In addition, the X- and Y-chromosomal genes were removed due to a significant gender difference between the stroke and asymptomatic patients.

Pairwise p-values for probe sets were calculated by nonparametric Mann-Whitney U-test. False discovery rate was estimated by using Significance Analysis of Microarrays (SAM) [2], a permutation-based variance-shrinkage method. We used a value of 0.539 for the tuning parameter delta, which corresponds to a median false discovery rate of 0.05. The following values were used for other adjustable parameters: response format - two class unpaired, minimum fold-change - 1.5, test statistic - Wilcoxon rank-sum test, number of permutations - 1000.

In addition to RMA, the hybridization data was also pre-processed by two other commonly used algorithms, GC-RMA [3] and MAS5 (http://www.affymetrix.com). Pre-processing was followed by otherwise similar filtering steps as for RMA-processed data, but the raw signal cut-offs were adjusted to 100 for GC-RMA and 200 for MAS5 based on hybridisation data on a technical replicate (see methods above). Genes with significantly differing expression levels between ACPs and SCPs at 5% FDR were again identified by SAM using the same values for the adjustable parameters as in the case of RMA. The three probe lists where combined in Venn diagram to get the intersection of the lists.

Hierarchical clustering was performed with GeneSpring v.7.3 (Silicon Genetics) using Pearson uncentered as the distance metric and centroid as the linkage rule. Gene enrichment analysis for Gene Ontology (GO) categories and functional pathways (KEGG and Biocharta) was performed for the probes showing ≥1.5-fold expression change (n=103) using WebGestalt software [4]. We used a prestored gene set (WebGestalt_HG_U133A) as a reference set and applied a hypergeometric test to identify enriched GO categories and pathways. The default significance level of 0.01 was used and a minimum number of genes was set as two. For more information on Gene-Ontology see http://www.geneontology.org/.

**Quantitative Real-Time PCR**

QPCR was performed using TaqMan® Gene Expression Assays (Supplemental Table 1) and the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s recommendations. Briefly, mRNA was converted to cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time qPCR reactions were performed in a 25 ul mixture of 20 ng of cDNA, 1x TaqMan Universal Master Mix and 1x Assay Mix containing primers and TaqMan probe (all from Applied Biosystems). Each sample was run in triplicate and only Ct values with standard deviation ≤ 0.16 were accepted. Each plate contained negative controls (no-template-controls) and the calibrator sample.
Prior to the analyses, suitable endogenous control genes for qPCR were determined by screening the expression of 11 commonly used endogenous control genes using a TaqMan® Human Endogenous Control Plates (Applied Biosystems). Beta-actin showed the most constant expression levels within ΔC_T ≤ +/-0.5 (Supplemental Figure 2).

Gene expression was determined by the comparative C_T- or the relative standard curve method. For each assay a delta-delta-Ct validation experiment was carried out. If the absolute value of the slope of log input amount vs. deltaCt was less than 0.1, data analysis was done by delta-delta-Ct method (Applied Biosystems User Bulletin no. 2 P/N 4303859B). Otherwise, the relative standard curve method was used (see Supplemental Table 1).

Supplemental Table 1. QPCR assays and the data analysis method

| Gene Name                                      | Gene Symbol | Assay ID^a  | Method^b |
|------------------------------------------------|-------------|-------------|----------|
| 1 CD36 molecule                                | CD36        | Hs01567191_m1 | Comparative |
| 2 CD163 molecule                               | CD163       | Hs00174705_m1 | Comparative |
| 3 Fatty acid binding protein 4                 | FABP4       | Hs00609791_m1 | Standard curve |
| 4 Perilipin 2                                  | PLIN2       | Hs00605340_m1 | Comparative |
| 5 Glutamate-ammonia ligase                     | GLUL        | Hs00374213_m1 | Standard curve |
| 6 Chemokine (C-C motif) ligand 18              | CCL18       | Hs00268113_m1 | Comparative |
| 7 Fucosidase, alpha-L-1, tissue                | FUCA1       | Hs00609173_m1 | Comparative |
| 8 Interleukin 1 receptor antagonist            | IL1RN       | Hs00893626_m1 | Standard curve |
| 9 Heme oxygenase (decycling) 1                | HMOX1       | Hs00157965_m1 | Comparative |
| 10 S100 calcium binding protein A8             | S100A8      | Hs00374263_m1 | Comparative |
| 11 Chemokine (C-X-C motif) receptor 4          | CXCR4       | Hs00976734_m1 | Comparative |
| 12 Mannose receptor, C type 1                 | MRC1        | Hs00267207_m1 | Comparative |
| 13 Heat shock 70kDa protein 6                  | HSPA6       | Hs00275682_s1 | Comparative |
| 14 Oxysterol binding protein-like 8            | OSBPL8      | Hs00970777_m1 | Comparative |
| 15 Apolipoprotein E                            | APOE        | Hs00171168_m1 | Comparative |
| 16 ADP-ribosylation factor-like 4C             | ARL4C       | Hs00255039_s1 | Comparative |
| 17 Regulator of G-protein signalling 5         | RGS5        | Hs01555176_m1 | Standard curve |
| 18 Myosin, heavy chain 10, non-muscle          | MYH10       | Hs00992055_m1 | Comparative |

^aTaqMan® Gene Expression Assays, Applied Biosystems (www.appliedbiosystems.com)

^bAll genes were normalized to beta-actin.
### Supplemental Table 2. Antibodies.

| Antigen                          | Clonality/clone<sup>a</sup> | Working concentration<sup>b</sup> | Manufacturer    |
|----------------------------------|------------------------------|-----------------------------------|-----------------|
| **Cell type markers**            |                              |                                   |                 |
| HAM56 (macrophage)               | PC                           | 0.71 µg/mL                        | Dako            |
| CD45RO (activated T-cell)        | MC/UCHL-1                     | 4.20 µg/mL                        | Dako            |
| Tryptase (mast cell)             | MC/G3                        | 0.12 µg/mL                        | Chemicon        |
| α-smooth muscle cell actin       | MC/1A4                        | 7.6 µg/ml                         | Dako            |
| **Replicated genes<sup>c</sup>** |                              |                                   |                 |
| CD36                             | PC                           | 2 µg/ml                           | Chemicon        |
| CD163                            | PC                           | 10 µg/ml                          | R&D Systems     |
| FABP4                            | PC                           | 0.3 µg/ml                         | Sigma           |
| PLIN2                            | MC/AP125                      | 1:100                             | American Research Products |
| GLUL                             | PC                           | 5 µg/ml                           | BD Biosciences Europe |
| CCL18                            | PC                           | 25 µg/ml                          | R&D Systems     |
| IL1RN                            | PC                           | 2.5 µg/ml                         | R&D Systems     |
| HMOX1                            | PC                           | 1:1500                            | Stressgen       |
| S100A8                           | PC                           | 2.5 µg/ml                         | Lifespan Biosciences |

<sup>a</sup>PC = polyclonal, MC = monoclonal. <sup>b</sup>Dilution is reported if antibody concentration is unknown. Mouse IgG1 and -3 (Dako), mouse IgG2a-b (Dako) mouse IgM (Dako), goat IgG (Zymed Laboratories Inc.) in equivalent dilutions or PBS were used as negative controls. <sup>c</sup>Antibodies tested against FUCA1 failed to produce a reliable staining pattern.
Supplemental Table 3. Microarray probe sets showing ≥ 1.5-fold change in expression between stroke-associated and asymptomatic CPs (n=103)

| Probe ID   | Gene Symbol | Gene Name                        | Fold-change | Mann-Whitney U-test p* | SAM q (%)* |
|------------|-------------|----------------------------------|-------------|------------------------|------------|
| 202206_at  | ARL4C       | ADP-ribosylation factor-like 4C  | 1.6         | 0.001                  | 1.72       |
| 213418_at  | HSPA6       | Heat shock 70kDa protein 6       | 1.8         | 0.001                  | 1.72       |
| 221760_at  | MAN1A1      | Mannosidase, alpha, class 1A, member 1 | 1.5       | 0.002                  | 1.72       |
| 210512_s_at | VEGF        | Vascular endothelial growth factor | 2.0      | 0.003                  | 1.72       |
| 200921_s_at | BTG1        | B-cell translocation gene 1, anti-proliferative | 1.6    | 0.004                  | 1.72       |
| 202207_at  | ARL4C       | ADP-ribosylation factor-like 4C  | 1.6         | 0.004                  | 1.72       |
| 212192_at  | KCTD12      | Potassium channel tetramerisation domain containing 12 | 1.6    | 0.004                  | 1.72       |
| 201670_s_at | MARCKS      | Myristoylated alanine-rich protein kinase C substrate | 1.6    | 0.006                  | 1.72       |
| 202998_s_at | LOXL2       | Lysyl oxidase-like 2             | 1.7         | 0.006                  | 1.72       |
| 203060_s_at | PAPSS2      | 3'-phosphoadenosine 5'-phosphosulfate synthase 2 | 1.5    | 0.006                  | 1.72       |
| 218149_s_at | ZNF395      | Zinc finger protein 395          | 1.5         | 0.006                  | 1.72       |
| 212372_at  | MYH10       | Myosin, heavy polypeptide 10, non-muscle | -1.7   | 0.007                  | 1.72       |
| 202499_s_at | SLC2A3      | Solute carrier family 2 (facilitated glucose transporter), member 3 | 1.7    | 0.009                  | 1.72       |
| 205099_s_at | CCR1        | Chemokine (C-C motif) receptor 1 | 1.6         | 0.009                  | 1.72       |
| 212154_at  | SDC2        | Syndecan 2                       | 1.8         | 0.009                  | 1.72       |
| 212334_at  | GNS         | Glucosamine (N-acetyl)-6-sulfatase | 1.5      | 0.009                  | 1.72       |
| 217028_at  | CXCR4       | Chemokine (C-X-C motif) receptor 4 | 1.8    | 0.009                  | 1.72       |
| 202310_s_at | COL1A1      | Collagen, type I, alpha 1        | 1.7         | 0.011                  | 1.72       |
| 212820_at  | DMXL2       | Dmx-like 2                       | 1.7         | 0.011                  | 1.72       |
| 221059_s_at | COTL1       | Coactosin-like 1                 | 1.6         | 0.011                  | 1.72       |
| 211404_s_at | APLP2       | Amyloid beta (A4) precursor-like protein 2 | 1.5    | 0.012                  | 1.72       |
| 201438_at  | COL6A3      | Collagen, type VI, alpha 3       | 1.7         | 0.013                  | 1.72       |
| 202679_at  | NPC1        | Niemann-Pick disease, type C1    | 1.5         | 0.013                  | 1.72       |
| 212959_s_at | GNPTAB      | N-acetylgalacosamine-1-phosphate transferase, alpha and beta subunits | 1.5    | 0.013                  | 1.72       |
| 222120_s_at | NPL         | N-acetylneuraminate pyruvate lyase | 1.8     | 0.013                  | 1.72       |
| 201193_at  | IDH1        | Isocitrate dehydrogenase 1 (NADP+), soluble | 1.6    | 0.016                  | 1.72       |
| 201744_s_at | LUM         | Lumican                          | 1.6         | 0.016                  | 1.72       |
| 202934_at  | HK2         | Hexokinase 2                     | 1.7         | 0.016                  | 1.72       |
| 213655_at  | YWHAE       | Tyrosine 3-monoxygenase          | 1.6         | 0.016                  | 1.72       |
| 215049_x_at | CD163       | CD163 molecule                   | 1.6         | 0.016                  | 1.72       |
| 217983_s_at | RNASET2     | Ribonuclease T2                  | 1.7         | 0.016                  | 1.72       |
| 218353_at  | RGS5        | regulator of G-protein signalling 5 | -1.7    | 0.016                  | 1.72       |
| 32128_at   | CCL18       | Chemokine (C-C motif) ligand 18   | 2.2         | 0.016                  | 1.72       |
| 203645_s_at | CD163       | CD163 molecule                   | 1.7         | 0.019                  | 1.72       |
| 209924_at  | CCL18       | Chemokine (C-C motif) ligand 18   | 2.0         | 0.019                  | 1.72       |
| 212670_at  | ELN         | Elastin                          | -1.7        | 0.019                  | 1.72       |
| Probe ID   | Gene Symbol* | Gene Name*                        | Fold-change | Mann-Whitney U-test * | SAM q (%)* |
|------------|--------------|-----------------------------------|-------------|-----------------------|-----------|
| 212671_s_at| HLA-DQA1 /   | Major histocompatibility complex,  | 1.8         | 0.019                 | 1.72      |
|            | HLA-DQA2 /   | class II, DQ alpha                |             |                       |           |
|            | LOC650946    |                                   |             |                       |           |
| 201645_at  | TNC          | Tenascin C                        | 1.6         | 0.023                 | 1.72      |
| 202838_at  | FUA1         | Fucosidase, alpha-L- 1, tissue    | 1.9         | 0.023                 | 1.72      |
| 209070_s_at| RGS5         | Regulator of G-protein signalling 5| -1.7        | 0.023                 |           |
| 209071_s_at| RGS5         | Regulator of G-protein signalling 5| -1.7        | 0.023                 |           |
| 212582_at  | OSBPL8       | Oxysterol binding protein-like 8  | 1.6         | 0.023                 | 1.72      |
| 213975_s_at| LYZ /        | Lysozyme                          | 1.7         | 0.023                 |           |
|            | LILRB1       |                                   |             |                       |           |
| 200638_s_at| YWHAZ        | Tyrosine 3-monooxygenase          | 1.5         | 0.028                 |           |
| 201170_s_at| BHLHB2       | Basic helix-loop-helix domain     | 1.5         | 0.028                 |           |
|            | class B, 2   |                                   |             |                       |           |
| 203814_s_at| NQO2         | NAD(P)H dehydrogenase, quinone    | 1.5         | 0.028                 | 1.72      |
| 204774_at  | EVI2A        | Ectropic viral integration site 2A| 1.5         | 0.028                 |           |
| 214770_at  | MSR1         | Macrophage scavenger receptor 1   | 1.5         | 0.028                 |           |
| 217202_s_at| GLUL         | Glutamate-ammonia ligase          | 1.7         | 0.028                 | 1.72      |
| 200648_s_at| GLUL         | Glutamate-ammonia ligase          | 1.9         | 0.033                 | 1.72      |
| 201200_at  | CREG1        | Cellular repressor of E1A-stimulated genes 1 | 1.5 | 0.033 | 1.72 |
| 202087_s_at| CTSL         | Cathepsin L                       | 1.5         | 0.033                 | 1.72      |
| 202436_s_at| CYP1B1       | Cytochrome P450, family 1, subfamily B, polypeptide 1 | 1.6 | 0.033 | 1.72 |
| 202859_x_at| IL8          | Interleukin 8                     | 1.9         | 0.033                 | 1.72      |
| 202902_s_at| CTSS         | Cathepsin S                       | 1.5         | 0.033                 | 1.72      |
| 204438_at  | MRC1 /       | Mannose receptor, C type 1        | 1.6         | 0.033                 | 1.72      |
|            | MRC1L1       |                                   |             |                       |           |
| 208146_s_at| CPVL         | Carboxypeptidase, vitellogenic-like| 1.5         | 0.036                 | 1.72      |
| 201147_s_at| TIMP3        | TIMP metallopeptidase inhibitor 3 | 1.9         | 0.039                 | 1.72      |
| 202266_at  | TTRAP        | TRAF and TNF receptor associated protein | 1.5 | 0.039 |           |
| 210889_s_at| FCGR2B       | Fc fragment of IgG, low affinity IIb, receptor (CD32) | 1.5 | 0.039 |           |
| 218559_s_at| MAFB         | V-maf musculoaponeuritic fibrosarcoma oncogene homolog B (avian) | 1.5 | 0.039 |           |
| 202345_s_at| FABP5 /      | Fatty acid binding protein 5      | 1.6         | 0.047                 | 1.72      |
|            | LOC653327    |                                   |             |                       |           |
| 204137_at  | GPR137B      | G protein-coupled receptor 137B   | 1.5         | 0.047                 |           |
| 206488_s_at| CD36         | CD36 molecule                     | 2.2         | 0.047                 | 1.72      |
| 212158_at  | SDC2         | Syndecan 2                        | 1.5         | 0.047                 |           |
| 219032_s_at| OPN3         | Opsin 3                           | 1.5         | 0.047                 |           |
| 219607_s_at| MS4A4A       | Membrane-spanning 4-domains, subfamily A, member 4 | 1.8 | 0.047 | 1.72 |
| 200766_at  | CTSD         | Cathepsin D                       | 1.5         | 0.055                 |           |
| 201669_s_at| MARCKS       | Myristoylated alanine-rich protein kinase C substrate | 1.6 | 0.055 |           |
| 202912_at  | ADM          | Adrenomedullin                    | 1.5         | 0.055                 | 1.72      |
| Probe ID    | Gene Symbol* | Gene Name*                      | Fold-change | Mann-Whitney U-test p* | q (%)* |
|------------|--------------|---------------------------------|-------------|------------------------|-------|
| 203665_at  | HMOX1        | Heme oxygenase (decycling) 1    | 2.2         | 0.055                  | 1.72  |
| 203980_at  | FABP4        | Fatty acid binding protein 4, adipocyte | 2.6         | 0.055                  | 1.72  |
| 212657_s_at | IL1RN        | Interleukin 1 receptor antagonist | 1.6         | 0.055                  | 1.72  |
| 214038_at  | CCL8         | Chemokine (C-C motif) ligand 8  | 1.6         | 0.055                  | 1.72  |
| 215223_s_at | SOD2         | Superoxide dismutase 2, mitochondrial | 1.5         | 0.055                  | 1.72  |
| 201212_at  | LGMN         | Legumain                        | 1.6         | 0.065                  |       |
| 201963_at  | ACSL1        | Acyl-CoA synthetase long-chain family member 1 | 1.5         | 0.065                  |       |
| 202381_at  | ADAM9        | ADAM metallopeptidase domain 9 (meltrin gamma) | 1.6         | 0.065                  |       |
| 204580_at  | MMP12        | Matrix metallopeptidase 12 (macrophage elastase) | 2.3         | 0.065                  | 1.72  |
| 209122_at  | PLIN2        | Perilipin 2                     | 1.8         | 0.065                  | 1.72  |
| 217294_s_at | ENO1         | Enolase 1                       | 1.7         | 0.065                  | 1.72  |
| 200832_s_at | SCD          | Stearoyl-CoA desaturase         | 1.6         | 0.076                  | 1.72  |
| 202283_at  | SERPINF1     | Serpin peptidase inhibitor, clade F | 1.5         | 0.076                  |       |
| 209351_at  | KRT14        | Keratin 14                      | 1.8         | 0.076                  | 1.72  |
| 209555_s_at | CD36         | CD36 molecule                   | 1.9         | 0.076                  | 1.72  |
| 203561_at  | FCGR2A       | Fc fragment of IgG, low affinity IIa, receptor | 1.5         | 0.088                  |       |
| 204446_s_at | ALOX5        | Arachidonate 5-lipoxygenase     | 1.5         | 0.088                  |       |
| 203504_s_at | ABCA1        | ATP-binding cassette, sub-family A (ABC1), member 1 | 1.5         | 0.102                  |       |
| 201785_at  | RNASE1       | Ribonuclease, RNase A family, 1 | 1.5         | 0.118                  | 3.26  |
| 202917_s_at | S100A8       | S100 calcium binding protein A8 | 1.6         | 0.118                  | 3.26  |
| 203936_s_at | MMP9         | Matrix metallopeptidase 9       | 1.6         | 0.118                  |       |
| 221730_at  | COL5A2       | Collagen, type V, alpha 2       | 1.5         | 0.136                  | 3.26  |
| 34210_at   | CD52         | CD52 molecule                   | 1.5         | 0.155                  |       |
| 201858_s_at | PRG1         | Proteoglycan 1, secretory granule | 1.5         | 0.177                  |       |
| 203381_s_at | APOE         | Apolipoprotein E                | 1.6         | 0.177                  | 4.00  |
| 217148_x_at | IGLV2-14     | Immunoglobulin lambda variable 2-14 | -1.7        | 0.177                  |       |
| 202075_s_at | PLTP         | Phospholipid transfer protein   | 1.5         | 0.201                  |       |
| 204259_at  | MMP7         | Matrix metallopeptidase 7       | 1.5         | 0.201                  | 4.72  |
| 203290_at  | HLA-DQA1     | Major histocompatibility complex, class II, DQ alpha 1 | -2.0        | 0.227                  |       |
| 216984_x_at | IGL@         | Immunoglobulin lambda locus     | -1.7        | 0.227                  |       |
| 211896_s_at | DCN          | Decorin                         | 1.5         | 0.256                  |       |
| 213831_at  | HLA-DQA1     | Major histocompatibility complex, class II, DQ alpha 1 | 2.0         | 0.320                  |       |
| 217022_s_at | IGHA1 / IGHA2 | Immunoglobulin heavy constant alpha 1 | 1.7         | 0.943                  |       |

*Genes are separated by hyphen if the probe detects several homologous genes, gene name is given for the first one. *p indicates the pairwise unadjusted p-values from nonparametric Mann-Whitney U-test, q the lowest FDR at which the gene is called significant from Significance Analysis of Microarrays (SAM).
## Supplemental Table 4. Enrichment of the genes showing ≥ 1.5-fold expression change (n=103) in Gene Ontology categories

| GO Category                      | Obs. # of genes | Exp. # of genes | p-value     | Gene Symbol                                                                 |
|----------------------------------|-----------------|-----------------|-------------|-----------------------------------------------------------------------------|
| **Biological Process**           |                 |                 |             |                                                                            |
| Catabolism                       | 9               | 3.62            | 0.0096      | MMP7, MMP9, GNS, RNASET2, LYZ, HMOX1, FUCA1, IDH1, HK2                      |
| Cholesterol homeostasis          | 2               | 0.06            | 0.0015      | NPC1, APOE                                                                 |
| Locomotory behavior              | 5               | 0.89            | 0.0019      | CCL8, CCL18, CCR1, IL8, VEGFA                                              |
| Organ development                | 12              | 4.13            | 0.0007      | DCN, SERPINF1, CYP1B1, ELN, COL1A1, COL6A3, IL8, MAFB, VEGFA, PAPSS2, MMP9, BTG1 |
| Physiological defense response   | 8               | 2.55            | 0.0037      | ALOX5, CCL8, CCL18, CD163, CCR1, S100A8, IL1RN, LYZ                         |
| Response to wounding             | 9               | 2.5             | 0.0008      | ALOX5, CCL8, CCL18, CD163, CCR1, S100A8, IL1RN, CD36                       |
| Physiological response to wounding| 8               | 2.42            | 0.0027      | ALOX5, CCL8, CCL18, CD163, CCR1, S100A8, IL1RN, CD36                       |
| Chemotaxis                       | 5               | 0.85            | 0.0015      | CCL8, CCL18, CCR1, IL8, VEGFA                                              |
| Inflammatory response            | 7               | 1.79            | 0.0020      | ALOX5, CCL8, CCL18, CD163, CCR1, S100A8, IL1RN                             |
| Regulation of neurogenesis       | 2               | 0.09            | 0.0038      | SERPINF1, APOE                                                              |
| Taxis                            | 5               | 0.85            | 0.0015      | CCL8, CCL18, CCR1, IL8, VEGFA                                              |
| Carbohydrate metabolism         | 8               | 2.79            | 0.0064      | MMP7, MMP9, MMP12, GNS, SLC2A3, FUCA1, IDH1, HK2                           |
| Cellular catabolism              | 9               | 3.06            | 0.0033      | MMP7, MMP9, GNS, RNASET2, LYZ, HMOX1, FUCA1, IDH1, HK2                      |
| Organ morphogenesis              | 6               | 1.72            | 0.0072      | DCN, SERPINF1, ELN, IL8, VEGFA, BTG1                                       |
| Sensory organ development        | 2               | 0.14            | 0.0082      | MAFB, CYP1B1                                                                |
| Vasculature development          | 4               | 0.59            | 0.0027      | SERPINF1, IL8, VEGFA, BTG1                                                 |
| Lipid transport                  | 4               | 0.47            | 0.0012      | PLTP, NPC1, OSBPL8, APOE                                                   |
| Positive chemotaxis              | 2               | 0.04            | 0.0007      | IL8, VEGFA                                                                 |
| Regulation of chemotaxis         | 2               | 0.06            | 0.0015      | IL8, VEGFA                                                                 |
| Response to reactive oxygen species| 2              | 0.09            | 0.0032      | SOD2, APOE                                                                 |
| Blood vessel morphogenesis       | 4               | 0.58            | 0.0026      | SERPINF1, IL8, VEGFA, BTG1                                                 |
| Blood vessel development         | 4               | 0.58            | 0.0026      | SERPINF1, IL8, VEGFA, BTG1                                                 |
| Cellular carbohydrate metabolism | 7               | 2.16            | 0.0060      | MMP7, MMP9, MMP12, GNS, FUCA1, IDH1, HK2                                   |
| Positive regulation of chemotaxis| 2               | 0.05            | 0.0011      | IL8, VEGFA                                                                 |
| Regulation of positive chemotaxis| 2               | 0.04            | 0.0007      | IL8, VEGFA                                                                 |
| Aminoglycan catabolism           | 2               | 0.03            | 0.0004      | FUCA1, GNS                                                                  |
| Angiogenesis                     | 4               | 0.56            | 0.0023      | SERPINF1, IL8, VEGFA, BTG1                                                 |
| Peptidoglycan metabolism        | 3               | 0.18            | 0.0007      | MMP7, MMP9, MMP12                                                           |
| Category                                      | Obs. # of genes | Exp. # of genes | \(p\)-value | Gene Symbol                   |
|----------------------------------------------|-----------------|-----------------|-------------|-------------------------------|
| Positive regulation of positive chemotaxis   | 2               | 0.04            | 0.0007      | IL8, VEGFA                   |
| Glycosaminoglycan catabolism                 | 2               | 0.03            | 0.0004      | FUCA1, GNS                   |
| Phosphate transport                          | 4               | 0.47            | 0.0013      | COL1A1, COL5A2, COL6A3, MSR1 |
| Induction of positive chemotaxis             | 2               | 0.04            | 0.0007      | IL8, VEGFA                   |
| Collagen catabolism                          | 2               | 0.15            | 0.0092      | MMP7, MMP9                   |

### Molecular function

|                                      | Obs. # of genes | Exp. # of genes | \(p\)-value | Gene Symbol                   |
|--------------------------------------|-----------------|-----------------|-------------|-------------------------------|
| Collagen binding                     | 2               | 0.13            | 0.0067      | LUM, MMP9                     |
| Peptidase activity                   | 9               | 3.38            | 0.0063      | MMP7, MMP9, MMP12, CPVL, CTSS, CTSD, CTSL, LGMN, ADAM9 |
| Sterol transporter activity          | 2               | 0.54            | 0.0012      | NPC1, ABCA1                   |
| G-protein-coupled receptor binding   | 3               | 0.38            | 0.0061      | CCL8, CCL18, IL8              |
| Endopeptidase activity              | 8               | 2.45            | 0.0030      | MMP7, MMP9, MMP12, CTSS, CTSD, CTSL, LGMN, ADAM9 |
| Scavenger receptor activity          | 3               | 0.17            | 0.0006      | LOXL2, CD163, MSR1            |
| C-C chemokine binding                | 2               | 0.13            | 0.0077      | CXCR4, CCR1                   |
| Chemokine activity                   | 3               | 0.31            | 0.0037      | CCL8, CCL18, IL8              |
| Chemokine receptor binding           | 3               | 0.32            | 0.0040      | CCL8, CCL18, IL8              |
| Metalloendopeptidase activity        | 4               | 0.66            | 0.0042      | MMP12, MMP7, MMP9, ADAM9      |
| C-C chemokine receptor activity      | 2               | 0.13            | 0.0077      | CXCR4, CCR1                   |

### Cellular Component

|                                      | Obs. # of genes | Exp. # of genes | \(p\)-value | Gene Symbol                   |
|--------------------------------------|-----------------|-----------------|-------------|-------------------------------|
| Extracellular space                  | 11              | 3.2             | 0.0003      | LOXL2, CCL18, MMP7, MMP9, IL8, ADM, VEGFA, S100A8, APOE, IL1RN, CCL8 |
| Extracellular matrix (sensu Metazoa) | 12              | 1.84            | \(2\times10^{-7}\) | LUM, DCN, TIMP3, MMP7, MMP9, MMP12, ELN, VEGFA, COL1A1, COL5A2, COL6A3, TNC |
| Collagen                             | 4               | 0.25            | 0.0001      | LUM, COL1A1, COL6A3, COL5A2  |
| Fibrillar collagen                   | 3               | 0.08            | \(5\times10^{-5}\) | LUM, COL1A1, COL5A2          |
| Vacuole                              | 8               | 1.06            | \(1\times10^{-7}\) | GNS, CTSD, CTSS, CTSL, GNPTAB, NPC1, FUCA1, LGMN |
| Lytic vacuole                        | 8               | 0.95            | \(4\times10^{-6}\) | GNS, CTSD, CTSS, CTSL, GNPTAB, NPC1, FUCA1, LGMN |
| Lysosome                             | 8               | 0.95            | \(4\times10^{-6}\) | GNS, CTSD, CTSS, CTSL, GNPTAB, NPC1, FUCA1, LGMN |

Gene enrichment analysis was performed for the probes showing \(\geq 1.5\)-fold expression change (n=103) using WebGestalt software [4]. For details, see Supplemental Methods. Categories below level 3, having two or more genes and enriched with a \(p\)-value < 0.01 are listed. Categories with significance level <0.001 are highlighted. Obs. # of genes = the number of observed genes in the category. Exp. # of genes = the expected number of genes in the category. For more information on Gene-Ontology see http://www.geneontology.org/.
Supplemental Table 5. Enrichment of the genes showing ≥1.5-fold expression change (n=103) in KEGG and Biocharta (BioC) pathways

| Pathway                                      | Source | Number of genes | p-value   | Gene Symbol                   | Gene Namea                                             |
|----------------------------------------------|--------|-----------------|-----------|-------------------------------|--------------------------------------------------------|
| ECM-receptor interaction (hsa04512)          | KEGG   | 6               | 1.9x10^-5 | COL1A1, COL5A2, COL6A3, TNC, SDC2, CD36 | Collagen types I alpha 1, V alpha 2 and VI alpha 3/ tenascin C/ syndecan 2/ CD36 |
| PPAR signaling pathway (hsa03320)            | KEGG   | 5               | 9.3x10^-5 | FABP4, ACSL1, PLTP, SCD, CD36 | Fatty acid binding protein 4/ acyl-CoA synthetase long-chain family member 1/ phospholipid transfer protein/ stearoyl-CoA desaturase/CD36 |
| Antigen processing and presentation (hsa0412) | KEGG   | 4               | 6.6x10^-4 | CTSL, CTSS, HLA-DQA1, LGMN | Cathepsins L and S/ major histocompatibility complex class II DQ alpha 1/ legumain |
| Inhibition of matrix metalloproteinases      | BioC   | 2               | 0.002     | MMP9, TIMP3                   | Matrix metallopeptidase 9/ TIMP metallopeptidase inhibitor 3 |
| Cytokine-cytokine receptor interaction (hsa04060) | KEGG   | 6               | 0.005   | CCR1, IL8, CCL8, CCL18, VEGF, CXCR4 | Chemokine (C-C motif) receptor 1/ interleukin 8/ chemokine (C-C motif) ligands 8 and 18/ vascular endothelial growth factor/ chemokine (C-X-C motif) receptor 4 |
| Cell communication (hsa01430)                | KEGG   | 4               | 0.005   | COL1A1, COL5A2, COL6A3, TNC, COL1A1, COL5A2, COL6A3, TNC, VEGF | Collagen types I alpha 1, V alpha 2 and VI alpha 3/ tenascin C/ vascular endothelial growth factor |
| Focal adhesion (hsa04510)                    | KEGG   | 5               | 0.009   | COL1A1, COL5A2, COL6A3, TNC, COL1A1, COL5A2, COL6A3, TNC, VEGF | Collagen types I alpha 1, V alpha 2 and VI alpha 3/ tenascin C/ vascular endothelial growth factor |

aHyphen separates genes. Gene enrichment analysis was performed for the probes showing ≥1.5-fold expression change (n=103) using WebGestalt software [4]. We used a prestored gene set (WebGestalt_HG_U133A) as a reference set and applied a hypergeometric test to identify enriched pathways. The default significance level of 0.01 was used and a minimum number of genes was set as two. For more information on KEGG see http://www.genome.ad.jp/kegg/ and on BioCharta see http://www.biocarta.com. Pathways are arranged according to p-value.
Supplemental Table 6. Correlations of qPCR confirmed expression changes with clinical data and plaque characteristics.

| Gene Symbol                  | CD36  | CD163 | FABP4 | PLIN2 | GLUL  | CCL18 | FUCA1 | IL1RN | HMOX1 | S100A8 |
|------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| **Clinical parameters**      |       |       |       |       |       |       |       |       |       |        |
| Gender                       | -0.28 | -0.17 | -0.14 | -0.36*| -0.35*| -0.22 | -0.31*| -0.04 | -0.27 | -0.10  |
| Degree of ICA stenoses       | 0.39* | 0.38* | 0.33* | 0.52**| 0.44* | 0.34* | 0.33* | 0.42* | 0.40* | 0.21   |
| **Laboratory measurements**  |       |       |       |       |       |       |       |       |       |        |
| Hematocrit                   | 0.19  | 0.19  | 0.11  | 0.35* | 0.23  | 0.26  | 0.22  | 0.15  | 0.06  | 0.01   |
| High-sensitivity CRP          | -0.12 | -0.09 | -0.19 | -0.23 | -0.18 | -0.16 | -0.06 | -0.21 | -0.24 | -0.14  |
| LDL-cholesterol              | 0.42* | 0.31  | 0.33  | 0.35  | 0.38  | 0.18  | 0.31  | 0.18  | 0.43* | 0.08   |
| HDL-cholesterol              | -0.16 | -0.18 | -0.01 | -0.15 | -0.03 | -0.26 | -0.16 | -0.00 | -0.05 | -0.04  |
| Fibrinogen                   | -0.09 | -0.01 | -0.03 | -0.05 | 0.02  | -0.07 | -0.04 | 0.11  | -0.04 | -0.30  |
| TPA antigen                  | 0.27  | 0.29  | 0.29  | 0.28  | 0.25  | 0.25  | 0.25  | 0.44* | 0.13  | 0.26   |
| **Macroscopic plaque characteristics** |       |       |       |       |       |       |       |       |       |        |
| Ulceration                   | 0.44* | 0.45* | 0.44* | 0.54**| 0.46* | 0.33* | 0.25  | 0.43* | 0.49**| 0.13   |
| Intraplaque hemorrhage       | 0.45* | 0.43* | 0.36* | 0.31  | 0.40* | 0.46* | 0.34* | 0.27  | 0.51**| 0.21   |
| Intramural thrombus          | 0.20  | 0.25  | 0.28  | 0.34* | 0.28  | 0.15  | 0.12  | 0.34* | 0.14  | 0.21   |
| Loose atheroma               | 0.11  | 0.02  | 0.07  | 0.04  | 0.20  | 0.05  | 0.18  | 0.27  | 0.04  | -0.08  |
| Calcification                | -0.35*| -0.20 | -0.30 | -0.22 | -0.33*| -0.33*| -0.39*| -0.32*| -0.32*| -0.08  |
| **Plaque components**a       |       |       |       |       |       |       |       |       |       |        |
| Macrophage density (HAM 56)(4) | 0.19  | 0.23  | 0.10  | 0.32  | 0.33  | 0.14  | 0.13  | 0.35* | 0.35* | 0.01   |
| T-cell density (CD45RO)(4)   | -0.04 | -0.01 | -0.10 | 0.12  | 0.06  | 0.08  | 0.07  | 0.11  | -0.07 | -0.22  |
| Mast cell density (anti-tryptase)(4) | 0.21  | 0.18  | 0.05  | 0.08  | 0.13  | 0.30  | 0.33  | -0.06 | 0.14  | -0.11  |
| Smooth muscle cells (alpha-actin) | -0.10 | -0.09 | -0.13 | -0.06 | -0.03 | -0.13 | 0.08  | -0.03 | -0.11 | -0.16  |
| **Markers of cell death and proliferation**a |       |       |       |       |       |       |       |       |       |        |
| Activated caspase 3(5)        | 0.44* | 0.38* | 0.31  | 0.42* | 0.51* | 0.44* | 0.55**| 0.37  | 0.36* | 0.24   |
| TUNEL positivity(5)          | 0.43* | 0.35* | 0.35* | 0.38* | 0.44* | 0.62**| 0.39* | 0.38* | 0.30  | 0.21   |
| Ki67 proliferation marker(5) | 0.39* | 0.41* | 0.29  | 0.51* | 0.50* | 0.28  | 0.31  | 0.44* | 0.48* | 0.17   |

The correlation coefficient reported is Spearman’s rho. Correlations were analyzed using all available data (n ranging from 24–43). Pairwise unadjusted p-values are denoted by * ≤ 0.05 or ** ≤ 0.001. Significant correlations (p<0.05) after Benjamini and Hochberg multiple testing correction are highlighted with grey. aImmunohistochemical analysis, immunostained antigen is specified in parenthesis if not apparent from the heading, the references to the original data are given in parenthesis.
Supplemental Figure 1. Representative electropherograms of mRNA samples from Bioanalyzer. On the y-axis are fluorescence units (FU) and on the X-axis time in seconds (s). S1 to S4 refer to four different mRNA samples extracted from carotid plaques. 18S and 28S refer to corresponding ribosomal RNAs.
Supplemental Figure 2. Evaluation of 11 endogenous control genes in the carotid plaque cDNA samples by qPCR. The y-axis (deltaCT (cycles)) shows the difference in the threshold cycle between the sample and a calibrator. One deltaCT equals to a twofold difference in initial template concentration. On the X-axis are tested control genes from left to right: IPC = internal plate control; 18S; HuPO (acidic ribosomal protein), huBA (beta-actin), huCYC (cyclophilin), huGAPDH (glyceraldehyde-3-phosphate dehydrogenase), huPGK (phosphoglycerokinase), huB2m (beta2-microglobulin), huGUS (beta-glucuronidase), huHPRT (hypoxanthine ribosyl transferase), huTBP (transcription factor IID, TATA binding protein) and huTfR (transferrin receptor). DeltaCT Sample 1 to Sample 3 refer to three different cDNA samples from carotid plaques. Beta-actin (huBA) was selected for the endogenous control gene because it showed least variability between samples.
Histological classification of the carotid plaques according to AHA by patient group and symptom. AHA class VIa lesions contain microscopic surface ulceration, VIb hematoma/hemorrhage, and VIc thrombotic deposit [5].
Supplemental Figure 4.

The amounts of macrophages, activated T-cells, and mast cells in ACPs and SCPs in the microarray and extended replication groups. Immunohistochemical analysis was done from the adjacent CP slice used for RNA extraction. The immunostained antigen is given in parenthesis. The results were analyzed using quantitative analysis of immunohistochemistry[6].
The amount of smooth muscle cells in ACPs and SCPs in the microarray and extended replication groups. Immunohistochemical analysis was done from the adjacent CP slice used for RNA extraction. The immunostained antigen is given in parenthesis.
Supplemental Figure 6. Venn diagram showing the number of significant probes common to different pre-processing algorithms. Hybridization data was pre-processed by either RMA, GC-RMA or MAS5 and filtered by using congruent criteria, after which genes that were differentially expressed at 5% false discovery rate were selected by SAM-analysis. This resulted in 60 probes for RMA, 77 for GC-RMA and 75 probes for MAS5. The three probe lists where combined in the Venn diagram to get the intersection of the lists. The 37 probes corresponding to 35 genes common to all three algorithms are shown in the box on the right. The gene symbols are listed in the descending (and from the leftward panel to the rightward panel) order of RMA fold-change (i.e. FABP4 has the highest fold-change when calculated from the RMA normalized data). The corresponding gene names can be found in Supplemental Table 3.
Directed acyclic graph of enriched gene ontology categories. The 3 main ontologies are shown in the upper row and further discriminated by background colour. Below each ontology, the categories enriched with a *p*-value <0.01 and including ≥3 genes (yellow background) and a *p*-value <0.001 (red outline) and the parental terms necessary for illustration are shown. Genes enriched in each category are listed in Supplemental Table 5. A gene can occur in one or more ontologies.
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