# Novel experimental therapies for atherosclerosis : a genomics based approach

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# Chapter 2

# Transcriptional Profiling of Initial Atherogenesis in LDL Receptor Deficient Mice Identifies Diet Induced Upregulation of FOXO1 controlled Genes

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## Abstract

Atherosclerosis is a multi-factorial disease associated with elevated plasma cholesterol levels, lipid deposition and inflammation within the vessel wall. Several molecules and transcriptional pathways have been identified in established atheroma, but initial factors contributing to the onset of disease are less well described.

In this study, we compared the transciptome of the aortic arch of LDL receptor deficient mice that were fed a Western type diet with chow fed mice by using two different statistical approaches. We identified transcription factor binding sites overrepresented within similarly regulated gene clusters and found that FOXO1 mediated transcription is enhanced during Western type diet feeding. This was further demonstrated by the significant upregulation of the FOXO1 downstream gene HMG-CoA reductase. In addition, we used Gene Set Enrichment Analysis to identify genes and pathways enriched in initial and early atherogenesis. We confirmed the specific upregulation of ABCB10 in initial lesion formation, and CCL8 in early lesion formation.

This study identifies new transcriptional regulators and genes associated with very early changes in the vascular wall that precede the large scale influx of leukocytes leading to atherosclerotic plaque formation. These new molecules might serve as targets to modulate the initiation of atherosclerotic lesions.

## Introduction

Atherosclerosis is a progressive disease of the medium to large arteries that is characterized by lipid deposition and a subsequent influx of leukocytes into the vessel wall[1]. It is the primary cause of myocardial infarction (MI) and stroke, and a major cause of death in the Western world[2]. Several risk factors have been identified over the past decade, including hyperlipidemia, smoking, hypertension, obesity and diabetes[3]. To increase the understanding of the disease, numerous studies have been performed that focused mostly on single genes, for example by using knockout animals, SNP analysis of a single gene or gene silencing via siRNA. Due to the multi-factorial and complex nature of atherosclerosis, a broad, non-biased approach will be highly informative, leading to a better understanding and insight in the biological processes that underlie the disease. Furthermore, not only genes that have previously been associated with atherosclerosis are included, but also unexpected genes that have never been linked to this disease can be identified.

Along this line, efforts have been made to identify disease related gene expression patterns in atherosclerosis via transcriptional profiling using micro-arrays[4]. Several studies are performed to define the transciptome of human atherosclerosis, but the identified genes were mostly linked to atherogenesis already[5-6]. They include genes involved in inflammation, lipid metabolism, matrix degradation as well as cell proliferation and differentiation. Due to the difficulties in human sample incorporation and isolation, mouse models for atherosclerosis provide an attractive alternative. The commonly used animal model for these studies is the ApoE deficient mouse. Array studies were performed to identify differentially expressed genes in mice of different ages, and mice fed with different atherogenic diets[7-11]. Functional gene groups that have been reported to be differentially expressed are chemokines and chemokine receptors, cathepsins and general pro-inflammatory markers.

The aim of the present study was to identify new genes and/or gene clusters that are differentially expressed during very early lesion formation in the aorta. For this purpose we used LDL receptor deficient ($LDLr^{-/-}$) animals, which unlike ApoE deficient animals, only develop atherosclerotic lesions when fed an atherogenic diet[12]. This provides the possibility to time the onset of disease, and identify very early changes in gene expression in the aortic wall. We used whole mount tissue from the aortic arch of animals at 0-1.5-3-4.5-9 weeks of Western type diet feeding and compared the transciptome to aortic tissue from mice on chow. By using advanced statistical analyses both on single transcript levels as well as on functional level we identified genes and pathways that are specific for changes in the vessel wall during the initiation of lesion formation.

## Material and methods

*Animal experiments*

Female LDLr$^{-/-}$ mice (n=6 per group) were fed an atherogenic Western type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Service, UK) for 0-1.5-3-4.5 and 9 weeks. After the indicated diet feeding periods, mice were anaesthetized, flushed with PBS and the aortic arch (thoracic) was prepared free of peri-adventitial fat tissue and snap frozen in liquid nitrogen. All animal experiments were approved by the Leiden University animal welfare committee and in concordance with Dutch law. Concentration of serum cholesterol was determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

*Micro-array protocol*

RNA was isolated from aortic tissue using the GTC method and DNAse treated. The RNA was linearly amplified for 1 round (starting material 1 µg total RNA) synthesizing anti-sense cRNAs with an average base length of 500 nucleotides using the Message-Amp kit from Ambion. Aminoallyl-UTP was incorporated with a molar ration of 1:1 to rUTP. Cy3 or Cy5 mono-reactive dyes were coupled according to the manufacturers' instructions (Amersham Bioscience, Piscataway, New Jersey). Labeled cRNA was purified using the RNeasy purification kit (Qiagen, Germany). All aortic RNA samples were hybridized against a common reference containing (in equal amounts and amplified one round); liver, spleen and aorta of LDLr$^{-/-}$ mice 6 weeks on western type diet and LDLr$^{-/-}$ mice treated with LPS, RAW cells treated with LPS, H5V cells treated with TNFα and Strategene universal mouse reference RNA.

Hybridization was performed on glass based micro-arrays representing 22.056 unique murine oligonucleotide sequences (Micro Array Department, University of Amsterdam, The Netherlands (http://www.microarray.nl/libraries.html).

Microarray data were acquired and imported in Rosetta Resolver database and Loess normalized (limma package, Bioconductor). Significant differences in time were calculated by using one-way Bayesian ANOVA with Benjamini-Hochberg multiple testing correction (Cyber-T, R script, UCLA) with genes detectable in all arrays and FDR < 25%.

Data were grouped into control (0 weeks of diet), no lesion formation, 1.5 and 3 weeks of diet), and initial lesion formation (4.5 and 9 weeks of diet). Hierarchical clustering of significant genes was performed using Cluster (Eisen Lab, UCB, http://rana.lbl.gov/EisenSoftware.htm)[13]. Conserved transcription factor binding sites in the region of 500 base pairs from ATG of genes overrepresented within the clusters were analyzed with whole genome rVista by using the 2004 mouse/human conserved genome database[14].

Genome wide analysis at group level was performed using GSEA after conversion of mouse to human signature[15]. The molecular signature database that was used was a modified version of the original named c2.symbols.gmt

(http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html) set and earlier described by Volger *et al*[16]. The dataset contained 594 gene sets. Analysis was performed with the following settings: 100 permutations on phenotype, gene sets with more that 10 genes were included in the analysis.

*Validation of candidate genes by RT-PCR*

Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (appendix I) were designed using Primer Express software with the manufacturer's default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

*Statistical analysis*

Values are expressed as mean ± SEM. Two-tailed student's T-test was used to compare normally distributed data. Mann-Whitney test was used to compare lesion area data and other not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

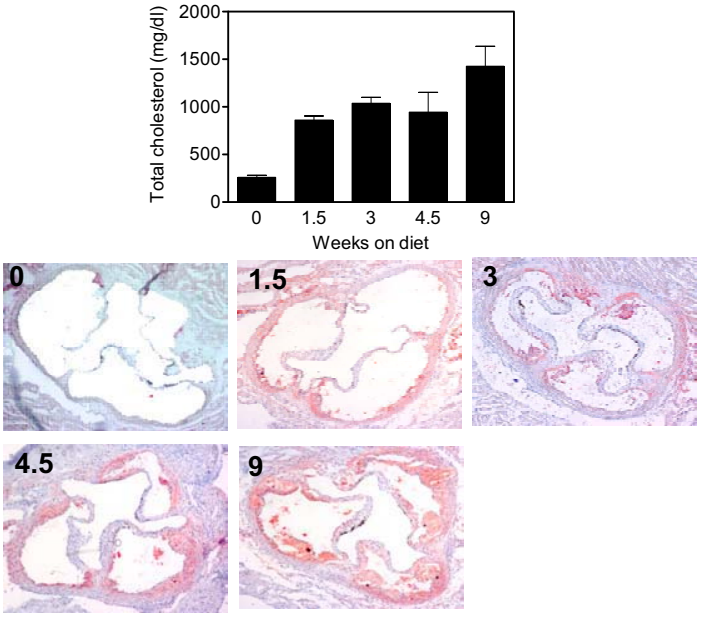


**Figure 1: Diet induced lesion formation in LDLr$^{-/-}$ mice.** Female LDLr$^{-/-}$ mice (n=6 per group) were placed on a Western type diet for 0-1.5-3-4.5 and 9 weeks. Total serum cholesterol was determined during the experiment and a significant increase is observed at 1.5 weeks of Western type diet feeding that is maintained throughout the experiment. Representative pictures of lesion formation in the aortic root at the indicated time points are visualized in the power panels. Oil red o staining is used to visualize lipids.

## Results

*Animal and tissue phenotype*

We designed this experiment to identify genes and genetic pathways that are specific for very early atherosclerotic lesion formation. We used LDLr$^{-/-}$ mice as a model for atherosclerosis as in this animal, the onset of lesion formation can be specifically timed by feeding a Western type diet. Female LDLr$^{-/-}$ mice (n=6/group) were placed on a Western type diet for 1.5; 3; 4.5; and 9 weeks. Chow fed animals were used as control. Western type diet feeding resulted in a significant upregulation of serum cholesterol levels already after 1.5 weeks from 260 ± 23 mg/dl to 862 ± 46 mg/dl. This increase in cholesterol level was constant throughout the experiment (Figure 1, upper panel). To determine the degree of atherosclerotic lesion formation, we stained the aortic valve leaflet area for lipid deposition using Oil-red-O. Representative photomicrographs are shown in figure 1 lower panels.

In general, the onset of lesion formation in the aortic valve leaflet area precedes plaque formation in the aortic arch. To provide insight in the degree of lesion formation in the aortic arch, we used real time PCR to check for the presence of macrophages and T cells.

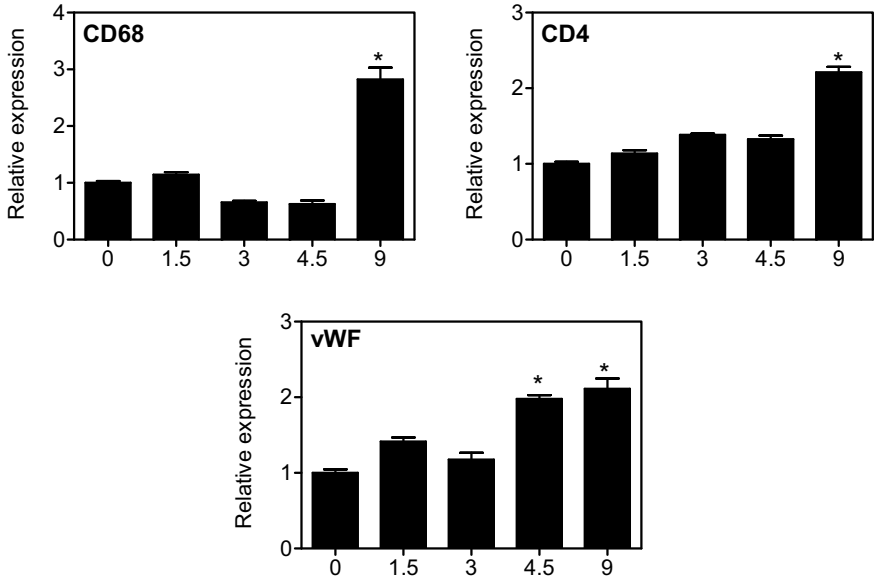


**Figure 2: Leukocyte influx and endothelial activation in the aortic arch.** We determined the extent of leukocyte influx within the aortic arch in time by RT-PCR on CD68 (macrophage specific) and CD4 (T cell specific) mRNA expression. Endothelial activation was assessed by vWF expression. mRNA was isolated from the aortic arch of LDLr $^{-/-}$ mice using the GTC method and expression of genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (*$p < 0.05$, n= 6 per time point).

Figure 2 shows that only after 9 weeks of Western type diet feeding, a significant upregulation of both CD68 mRNA (macrophage specific, 2.8 fold, p=0.023) and CD4 (T cell specific, 2.2 fold, p=0.02) was observed. This indicates that these leukocytes have entered the aortic wall and atherosclerotic plaque formation has been initiated. The activation of the endothelium that precedes the leukocyte influx is already visible after 4.5 weeks of Western type diet. This is shown by the significant upregulation of von Willebrand factor at 4.5 and 9 weeks of diet (2.0 fold, p=0.01, and 2.1 fold, p=0.03 respectively).

*Transcriptional regulation in the aortic arch during Western type diet feeding*

Transcriptional differences between the aortic arch of control mice and diet fed mice were determined by micro-array analysis. mRNA, isolated from every aortic arch was amplified one round, labeled with Cy5 and subsequently hybridized against a common reference (Cy3). Genes significantly regulated in time were identified by using Bayesian one-way ANOVA with Benjamini-Hochberg correction for multiple testing. Genes were considered differentially expressed when expression was observed in all the arrays, and the False Discovery Rate (FDR) < 25%. The resulting significant genes (N=354) were clustered using hierarchical clustering software. The time points were grouped in control tissue on chow diet resulting in low cholesterol levels (LC, 0 weeks), high serum cholesterol and no lesion formation (HC, 1.5 and 3 weeks,) and high cholesterol and initial lesion formation (HCIL, 4.5 and 9 weeks) and regulation profiles over these 3 situations were analyzed. The resulting clusters and gene lists are visualized in figure 3. Clustering resulted in 5 unique clusters as visualized in the following order; A: Induced HC and HCIL, N=76 genes; B: reduced HCIL only, N=19; C: induced HCIL only, N=16; D: reduced HC only, N=239; E: reduced HC and HCIL, N=10.

*Transcription factor binding sites overrepresented within the clusters*

Once we identified the above described differentially regulated gene clusters, we searched for coordinate regulation of these genes via possible common transcription factor binding sites in upstream regions of the genes present in each cluster by using rVISTA. We used the human-mouse alignment of May 2004 and searched within a 500 base pair upstream region of ATG. Distinct transcription factor binding sites were identified for each cluster and these are visualized in figure 3 right panel. In the genes that are present in the upper cluster, with induced expression in both high cholesterol situations, we found overrepresentation of transcription factor binding sites for FOXO1, GATA2, MEIS1, and c-myb. FOXO1 is involved several metabolic signaling pathways; overexpression of FOXO1 leads to hyperglycemia and hypertriglyceridemia, and exercise leads to downregulation of FOXO1 regulated genes[17-20]. GATA2 regulation is described for vascular endothelial and/or smooth muscle cells and Seo *et al.* have shown that GATA2 expression is one of the most predictive genes for human aortic sites with high lesion burden[5].

| A: Induced HC and HCIL: N=76 (61 without clones) | | | | | |
|---|---|---|---|---|---|
| Aadat | Cox7a1 | Gpd2 | Kel | Prr6 | Tead4 |
| Aatk | Ctgf | Gpr155 | Lcn5 | Ptp4a1 | Tex23 |
| Abca8b | Cyp2j9 | Gria1 | Lin7c | Retn | Tshb |
| Akap12 | Decr1 | Gsh2 | LOC545854 | Rpl27a | Ugt8a |
| Aldh1a7 | Ebf3 | Hadhb | Lum | Sardh | Vac14 |
| Arl4a | Ecgf1 | Igh-6 | Mrpl15 | Sh2d2a | Yipf1 |
| Btd | Eya4 | Igh-VS107 | Mylk | Ski | |
| Ccndbp1 | Fabp9 | Inpp4a | Nmral1 | Smarcd1 | |
| Cd164l2 | Fbxl22 | Irak1bp1 | Nppc | Smpdl3a | |
| Cdk5rap3 | Fga | Itga8 | Ogdh | Ssfa2 | |
| Chi3l1 | Fmo1 | Kcne4 | Ogn | Supt4h2 | |

| B: reduced HCIL only: N=19 (16 without clones) | | | | | |
|---|---|---|---|---|---|
| Ablim1 | C1qtnf3 | Fabp4 | Kpna2 | Uqcr | Zfp777 |
| Ap1m1 | D10Ertd610e | Gng10 | Oip5 | Usp10 | |
| Bub3 | Egfr | Hars | Pctp | Wbscr16 | |

| C: induced HCIL only: N=16 (14 without clones) | | | | | |
|---|---|---|---|---|---|
| Acss2 | D10Jhu81e | Lincr | Magmas | Prkar1a | Tacstd2 |
| Agpat3 | Gm1077 | Lpin1 | Nfat5 | Sox18 | Vps53 |
| Cyp2c38 | Il6st | | | | |

| D: reduced HC only: N=239 (210 without clones) | | | | | |
|---|---|---|---|---|---|
| Acp2 | Cops3 | Gmnn | Metap2 | Psma1 | Ssb |
| Acyp1 | Copz1 | Gnpnat1 | Mettl9 | Psma5 | St6gal1 |
| AK013368 | Cript | Gorasp2 | Mobk1b | Psmd10 | St7 |
| AK018642 | Crisp2 | Gpiap1 | MOU384-2 | Psmd3 | Stam |
| Ak3 | Csnk1g3 | Grina | Mphosph6 | Ptges | Stat2 |
| Ankrd11 | Cxcl1 | Gstt1 | Mrpl13 | Rab35 | Sugt1 |
| Apex1 | D4Wsu53e | Gtf2i | Mrpl49 | Rad21 | Sulf2 |
| Aplp1 | Dars | Hdgf | Mrps10 | Ranbp1 | Tbc1d10b |
| Apobec1 | Ddef1 | Hp | Mtdh | Rbm17 | Tbrg1 |
| Apof | Ddx5 | Hsd11b1 | Mttp | Rbx1 | Tdrkh |
| Apoh | Derl1 | Id2 | Mup1 | Rpl30 | Timm17b |
| Aqp2 | Dhx15 | Ifi204 | Mxi1 | Rpl38 | Tm9sf3 |
| Arhgef6 | Dnaja1 | Igj | Myh8 | Rps10 | Tmed4 |
| Arid1a | Drctnnb1a | Igk-V | Myo5a | Rps13 | Tmem165 |
| Asns | Eef1a1 | Igk-V19-14 | Nasp | Ryk | Tmpo |
| Aspm | Efemp2 | Igkv4-91 | Nbn | S100a4 | Top3b |
| Atg5 | Eif2ak3 | Inpp5b | Ndrl | Sap30bp | Trex1 |
| Azgp1 | Eif2b4 | Josd1 | Nktr | Serpina1b | Ttc18 |
| BC005512 | Eif3s5 | Josd3 | Nol5 | Serpine1 | Ttn |
| Bgn | Eif4g2 | Kifc3 | Npm1 | Set | Tubb2b |
| Birc5 | Elk3 | Klf10 | Oat | Setdb1 | Txnrd1 |
| Bxdc2 | Emcn | Krr1 | Omt2b | Sfrs1 | U86712 |
| Calu | Eno2 | Larp-pending | Pacsin2 | Sgk2 | Ubb |
| Cast | Eprs | Lgals9 | Pbk | Slc35a3 | Ube2c |
| Cct4 | Ergic2 | Lilrb4 | Pdpn | Slc38a4 | Ubl4 |
| Cd53 | Esd | LOC280487 | Pef1 | Slc7a6os | Ubp1 |
| Cdt1 | Fbln2 | LOC280487 | Phc2 | Slco1b2 | Ulk2 |
| Cebpg | Fbxo7 | LOC544945 | Phf3 | Slpi | Wac |
| Cenpa | Fkbp1a | LOC545854 | Pias1 | Smarcd1 | Wars2 |
| Cetn3 | Fscn1 | LOC629915 | Piga | Smu1 | Xlr3a |
| Chia | Ftl1 | Lsm5 | Pja2 | Snrpe | Xpo1 |
| Chm | Gc | Lsm8 | Pla2g4a | Snx1 | Zfand1 |
| Chuk | Gcap27 | M17761 | Prkcd | Snx5 | Zfand5 |
| Cited2 | Glud1 | Map3k5 | Prpf40a | Spsb3 | Zfp58 |
| Cks2 | Gm1419 | Mark3 | Prss23 | Sptlc1 | Zfpm1 |

| D: reduced HC and HCIL: N=10 (8 without clones) | | | | |
|---|---|---|---|---|
| | Apex1 | Id2 | Rab35 | Timm17b |
| | Asns | Piga | Rpl38 | U86712 |

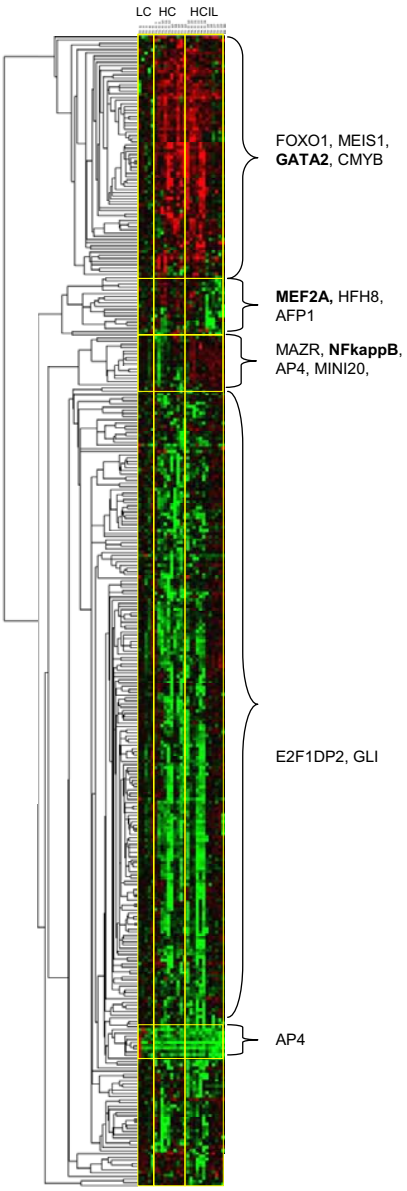


**Figure 3: Differentially regulated genes during diet induced lesion formation.** Differential regulation due to Western type diet feeding in the aortic arch was assessed by Bayesian one-way ANOVA with BHB correction and 354 genes were found to be significantly differentially expressed in time. Hierarchical clustering of these significant genes resulted in 5 distinctly regulated clusters as shown in by }. Presence of overrepresented TFBS in the promoter region of the genes in each cluster was identified by comparison with the 2004 mouse/human conserved genome database by using rVista. For each cluster these transcription factors are shown. Transcription factors that have been previously associated with vascular biology are shown in bold. **LC**: aortic arch from mice on chow; **HC**: aortic arch from mice on high cholesterol diet, no plaque formation; **HCIL**: aortic arch from mice on high cholesterol diet, initial lesion formation.

Next to this finding, 5 polymorphisms in the GATA2 gene were shown to be correlated to coronary artery disease[5,21]. MEIS1 is mainly associated with leukemogenesis and lymphocyte cell differentiation during early life[22]. C-myb was initially described as proto-oncogene, but some evidence exists that is functions as a mediator of vascular smooth muscle cell proliferation and neo-intima formation[23-26].

In initial lesion formation, when initial leukocyte infiltration has started, we observed an expected upregulation of genes regulated by the inflammatory regulator NFkappaB. This transcription factor has been extensively researched in the context of atherogenesis, and induces the expression of adhesion molecules, chemokines and matrix-metalloproteinases[26,27]. Other transcription factor binding sites that are enriched within this time point are MAZR and AP4, both with no direct described function in vascular cells until now.
Interestingly, during initial lesion formation, genes with a MEF2 and HFH8 (FOXF1) transcription factor binding site are downregulated. MEF2 regulation is associated with both endothelial and smooth muscle cell function[28],[29]. Furthermore, a mutation in MEF2a is linked to coronary artery disease[30]. HFH8 is less well described, but has been linked to vascular endothelial growth factor (VEGF) expression and LKLF mediated regulation during lung development[30].
The largest gene cluster contains genes that were reduced in both high cholesterol no lesions and high cholesterol with initial lesions. The overrepresented transcription factor binding sites are E2F1DP2 and GLI. E2F1DP2 has no downstream genes that are functionally described. GLI however, has been studied in more detail and is reported as a mediator of hedgehog signaling in oncogenic pathways leading to a variety of lethal tumors [31]. The last cluster contains genes that are downregulated during Western type diet feeding, both in initial and early atherogenesis, and shows enrichment for AP-4 binding sites. This transcription factor was also associated with an upregulation of genes in early atherogenesis.

*The expression of HMG-CoA reductase is upregulated during Western type diet feeding*

Recent reports link the expression of 3-hydroxy 3-methylglutaryl coenzyme A (HMGCoA) reductase directly to the level of active FOXO1[17]. In order to functionally validate the observed enrichment of FOXO1 induced genes after Western type diet feeding, we analyzed the expression of HMGCoA reductase in our samples. We observed a clear and significant upregulation of this gene due to Western type diet administration and this regulation profile correlates with the cluster profile (Fig 4). This indicates that FOXO1 downstream gene expression is enhanced in the aortic wall exposed to elevated cholesterol levels.

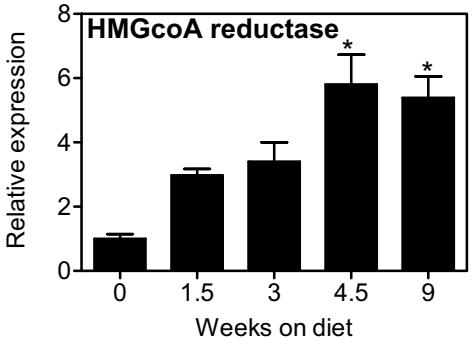


**Figure 4: Expression of HMG Co-A reductase in the aortic arch on Western type diet feeding.** mRNA was isolated from the aortic arch of LDLr$^{-/-}$ mice using the GTC method and expression of genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (*$p < 0.05$, n= 6 per time point).

*GSEA*

Next to the gene based pattern analysis, we performed pathway based Gene Set Enrichment Analysis to study transciptome differences in the aortic arch of initial and early lesion formation. This method enables the interpretation of genome-wide expression profiles based on biological function. Next to the functional output, this analysis tool identifies a top 100 of signature genes of one situation compared to another[15]. An enrichment score and relevant p value is calculated based on the ranked difference in expression of genes between two situations. In this way, gene sets that are overrepresented at the extremes of the ranked gene list will result in high enrichment scores. Tables 1 A and B show the enriched pathways for both initial and early lesion formation, and the nominal P values calculated by weighted Kolmogorov-Smirnov-like statistics as described earlier[15].

Immediately after Western type diet feeding (1.5 and 3 weeks of diet) a clear enrichment of metabolism associated genes is observed compared to early atherosclerosis (upper table). However, none of these sets were significantly enriched. In initial atherosclerosis (4.5 and 9 weeks of diet), the number of significant ($p<0.05$) enriched gene sets is 24. Next to metabolism associated sets, cell adhesion and map kinase activation pathways are observed.

Figure 5 shows the top 100 signature genes (50 top, 50 bottom) according to the intensity differences between initial and early atherosclerosis.

We used real-time PCR to validate genes that were present in the gene list generated by GSEA. ABCB10 expression is one of the top enriched genes in the high cholesterol, no lesion time point, indicating a diet induced effect on its regulation. ABCB10 is a relatively new member of the ABC binding cassette transporter family and has not been linked to atherogenesis yet. A significant upregulation of ABCB10 expression is observed after 3 weeks of Western type diet.

CCL8 (formerly known as MCP-2) is one of the top ranked genes in early atherosclerosis. CCL8 functions as a chemotactic factor for monocytes via CCR1 and CCR2b[32-35]. Real-time PCR showed that CCL8 expression indeed increased markedly by 2.4 fold ($p=0.02$) in aortic tissue with early lesions.

| High Cholesterol | NOM p-val |
|---|---|
| MAP00330_Arginine_and_proline_metabolism | 0.067 |
| MAP03020_RNA_polymerase | 0.070 |
| GO_ROS | 0.166 |
| MAP00240_Pyrimidine_metabolism | 0.288 |
| MAP00220_Urea_cycle_and_metabolism_of_amino_groups | 0.322 |
| no1Pathway | 0.176 |
| MAP00562_Inositol_phosphate_metabolism | 0.278 |
| MAP00910_Nitrogen_metabolism | 0.333 |
| hdacPathway | 0.321 |
| INSULIN_2F_DOWN | 0.308 |

| Initial lesion | NOM p-val |
|---|---|
| fatty_acid_metabolism | <0.001 |
| Eicosanoid_Synthesis | <0.001 |
| MAP00590_Prostaglandin_and_leukotriene_metabolism | <0.001 |
| SIG_Regulation_of_the_actin_cytoskeleton_by_Rho_GTPases | 0.018 |
| Wnt_Signaling | 0.018 |
| cell_adhesion_molecule_activity | 0.019 |
| tnfr2Pathway | 0.019 |
| GLUCO | 0.020 |
| MAP00052_Galactose_metabolism | 0.021 |
| electron_transporter_activity | 0.025 |
| MAP00710_Carbon_fixation | 0.027 |
| ST_GRANULE_CELL_SURVIVAL_PATHWAY | 0.030 |
| GLYCOL | 0.037 |
| KRAS_TOP100_KNOCKDOWN | 0.041 |
| GLUT_UP | 0.042 |
| mRNA_splicing | 0.043 |
| mprPathway | 0.048 |
| ST_Wnt_Ca2_cyclic_GMP_Pathway | 0.056 |
| SIG_PIP3SIGINCARDIACMYOCTES | 0.057 |
| wntPathway | 0.058 |
| ca_nf_at_signalling | 0.062 |
| CR_PROTEIN_MOD | 0.063 |
| cell_adhesion_receptor_activity | 0.064 |
| ST_Tumor_Necrosis_Factor_Pathway | 0.068 |

**Table 1: enriched gene sets identified using GSEA.** GSEA was used on our data set to identify differential pathways in week 1.5-3 (high cholesterol and initial lesion formation (week 4.5-9) in the aortic arch. GSEA identified no significant pathways in the initial stage, but 24 pathways were significantly enriched in the initial lesion formation stage. Next to pathways associated with metabolism, 4 immunological pathways are identified (indicated in gray).

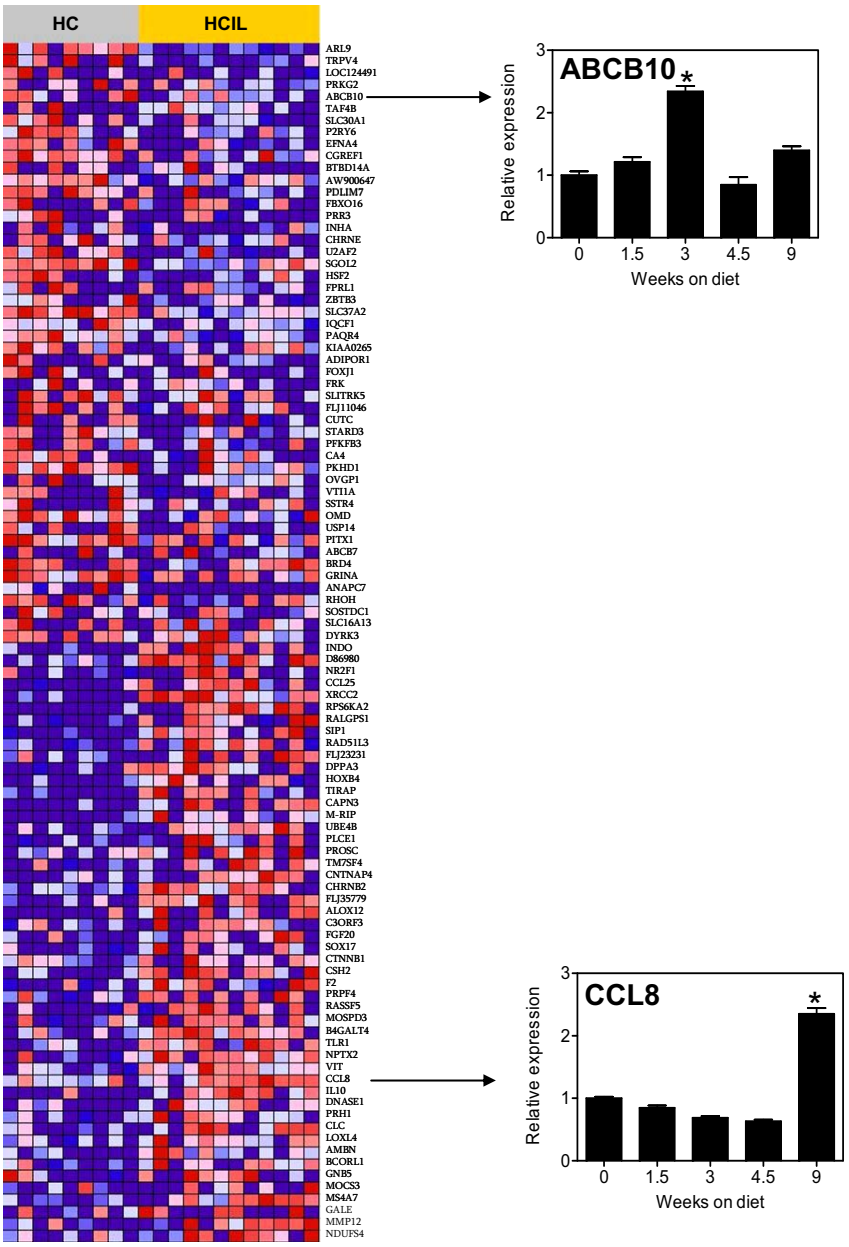


**Figure 5: Ranked gene list of top 100 most differential genes between initial and early lesion formation.** GSEA analysis uses a ranked gene list of expression differences between initial and early lesion formation to identify the above described pathways. A heat map of the 100 most differentially regulated genes, 50 from initial, and 50 from early lesion formation, is shown. Verification of some of these candidates was performed by RT-PCR and the resulting expression profiles are shown in the right panels.

## Discussion

In the present study we compared gene expression profiles from vascular tissue of LDLr$^{-/-}$ mice on different times of diet. We used whole mount material of the aortic arch, consisting of endothelial cells, smooth muscle cells and when plaque formation has started, leukocytes. This leukocyte influx was clearly demonstrated after 4.5 weeks and was significantly present at 9 weeks of diet feeding, as the mRNA levels of CD68 and CD4 were elevated at week 9. Furthermore, we showed that endothelial activation was already present at 4.5 weeks of Western type diet by increased expression of vWF[36]. Based on these observations we decided to group our arrays in control (chow fed animals), high cholesterol and no lesions (1.5 and 3 weeks of diet) and initial lesion formation (4.5 and 9 weeks). In this way, we enlarged the statistical power of our analysis and created the opportunity to compare aortic tissue from mice on diet without any lesion with very early lesions. We used two statistical approaches to find relevant transciptome differences between the time points, because no straight forward analysis pipeline exists for the analysis of time dependent microarray data. These two methods, one-way ANOVA followed by transcription factor binding site scan and GSEA pathway analysis are discussed separately below.

*Transcription regulation during initial and early lesion formation*

From the transcription factor binding site analysis, we identified FOXO-1 as a new possible regulator of diet induced vascular transcription. Genes regulated by this transcription factor are specifically overrepresented in the cluster with genes that are upregulated both in initial as well as in early lesion formation. This transcription factor expression inside the nucleus is regulated by phosphorylation by Akt and AMPK, as phosphorylation results in rapid degradation. Fissltaler *et al*. show that shear stress resulted in rapid phosphorylation FOXO1 and that its downstream target HMGCo-A reductase is subsequently decreased[17]. Next to this, FOXO1 has been shown to mediate responses in diabetes, glucose metabolism and exercise[18-20]. We show in our study, that diet induced genes are regulated by FOXO1 and that subsequently, its downstream target HCR is significantly upregulated in the aortic arch during Western type diet feeding. This is an interesting observation and indicates that FOXO1 is a mediator of both shear stress, as shown by Fissltaler et *al*, and diet induced alterations in vascular transcription. Pro-atherogenic conditions such as low shear stress, high glucose or lipid levels and little exercise all increase FOXO1 mediated transcription[17-20]. Therefore FOXO1 provides an interesting switch mechanism uniting the pro- atherogenic effects of several identified risk factors in atherogenesis.

Furthermore, we identified specific transcription factors for initial lesion formation. At this point, a moderate influx of leukocytes was confirmed by increased mRNA expression of both CD68 and CD4. Genes with a TFBS for

MEF2 were significantly overrepresented in the gene set that was downregulated at this time point. In contrast, genes with a TFBS for the inflammation associated transcription factor NFkappaB were overrepresented in the cluster with upregulated genes. MEF2 is a transcription factor that has been mostly associated with muscle development but is currently investigated in endothelial cell biology[28,37]. A mutation of the MEF2A gene is associated with increased risk for coronary artery disease[30]. Interestingly, MEF2 activity is inversely correlated with NFkappaB in our aortic material with early lesions. Kumar *et al.* have described a similar activity regulation profile in TNFα stimulated endothelium. The pro-inflammatory mediator TNFα downregulates the expression of KLF2, and this decrease is mediated by inhibition of MEF2 by NFkappaB[38]. We propose that a similar regulatory mechanism is activated in vascular tissue in a high fat environment leading to atherosclerotic plaque formation.

*Pathways and genes identified by GSEA*

In the current study we have found some interesting new target genes that were overexpressed in aortic tissue with initial or early atherosclerosis. In the top 50 most differentially regulated genes in initial lesion formation we identified 2 ABC transporters namely ABCB7 and ABCB10. ABCB10 is a member of the MDR/TAP subfamily, which members are associated with multi-drug resistance. Until now, the function of this mitochondrial protein is unknown, but a role in antigen presentation is suggested[39,40]. We confirmed the upregulation of ABCB10 by RT-PCR and identified this molecule as initially elevated due to Western type diet feeding.

CCL8, belonging to the small inducible cytokine family, was identified by GSEA as one of the top most differentially expressed within initial atherosclerosis. Real time PCR confirmed these findings by GSEA. Several chemokines, such as CCL5 and CCL2 have been shown to be upregulated in atherogenesis, and specific blockade or downregulation of these chemokines has been shown to be protective in atherogenesis[8, 41-44]. CCL8 functions as an attractor for monocytes via the CCR2 receptor. Unlike the receptor CCR2, the ligand CCL8 has never been associated with (early) atheroma so far. Functional blockade of this (possibly endothelial derived) molecule in animal models for atherosclerosis is necessary to address its now theoretical role in lesion formation.

We conclude that leukocyte influx into the vessel wall to initiate plaque formation is preceded by regulation of genes which are transcriptionally regulated by FOXO1. When the fist leukocytes enter the vessel wall, NFkappaB regulation is enhanced, combined with a decrease in MEF2 associated transcription. Our study thus identifies new transcriptional pathways and genes that underlie the early changes in the vessel wall, ultimately leading to atherosclerotic plaque formation.

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