

ATHERO-Express

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Executive Summary

ATHERO-EXPRESS is an initiative for early stage **drug target screening** in the field of cardiovascular diseases and for screening of **potential collateral side effects of any drug target** in diseased vascular tissues. In ATHERO-EXPRESS we have collected a large data repository containing tissue (plaques) and data of a large number of atherosclerosis patients. Geno- and phenotyping data of these patients are available, and the data set will be expanded continuously by further analyses. Collaborating partners will be able to request analysis of drug targets of their choice. ATHERO-EXPRESS will report on gene-phenotypical associations on transcriptomic, genomic and epigenomic levels. Expression patterns are presented for bulk plaque material as well as for each individual vascular cell type. Subsequently, the association of such variants with plaque characteristics and patients' clinical characteristics will be investigated. This will yield a wealth of data on expression, role and function of the drug target, to support decision-making in drug development for the private partners.

Management Team

The coordinators of ATHERO-EXPRESS are experienced investigators from the University Medical Centre Utrecht. They have extensive experience in biobanking and analysis of genetic and transcriptomic data.

Business Idea and Technology

The partners have set up plaque biobanks with ~4000 atherosclerotic lesions from carotid, femoral and iliac arteries. Histological plaque characteristics of and clinical patient data are included. Large sample numbers have been genotyped using genome-wide single-nucleotide polymorphism (SNP) arrays (n=2200) and 700 overlapping samples were analyzed for DNA methylation in atherosclerotic carotid plaques using the Illumina Methylation 450K array. From the same sample set we have executed bulk RNA sequencing of 700 samples. **In addition, in an ongoing study we generated single cell plaque RNA** sequencing of 50 plaques. In addition, genotyping data of monocytes and stored cell fractions of 500 patients undergoing coronary catheterization, expression data in tissues relevant in atherosclerotic disease, and data on >40 immunologically relevant proteins in carotid lesions are available. The substantial overlap between these data types facilitates the discovery of expression, methylation and protein quantitative trait loci (eQTL, mQTL, and pQTLs respectively).

Planning & Realization

Next to the existing (epi)genetic data, ATHERO-EXPRESS is performing quantitative proteome analyses to provide further information on tissue protein levels. In addition, analysis of cytokine and chemokine expression and micro-array analysis of blood cells will yield information on the role of the immune system. This initiative can thus access deep geno- and phenotyping data to provide partners with reports on proposed drug targets. The planning and priorities for further phenotyping will be decided in consultation with collaborative partners.

Market

By screening tissue and cell specific expression of proposed drug targets at an early stage against data from patients' samples, relevant genetic and phenotypic associations of any target can be examined before entering clinical trials. This will facilitate early decisions by pharma companies on which drugs are more likely to advance to (pre-) clinical development and ultimately reduce failure of clinical drug trials.

Cost & funding

We have planned deep phenotyping and transcriptomic studies as described above in order to extend the data available. To perform these studies and generate drug target association reports, we are therefore seeking to enter into collaborations with interested private companies, who will get the opportunity to request association studies on their drug targets of choice in the database and to further explore the data in depth.

ATHERO-EXPRESS

a. The consortium

ATHERO-EXPRESS provides the opportunity to perform powerful association studies to screen cardiovascular drug targets in an early stage of development. ATHERO-EXPRESS offers an extensive screening package including

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clinical, (epi)genetic, histological, transcriptomic, and proteomics data of tissues from patients with atherosclerosis. Through early screening of drug targets in an extensive data repository, the ATERO-EXPRESS consortium aims to improve the success of cardiovascular drug development.

b. Needs in (cardiovascular) drug discovery

Many pharmaceutical companies are developing novel CVD drug candidates, but the failure rate, even in late stage trials, is high. For example, in the cardiovascular field, 137 drugs failed to reach the market over the last 10 years after being launched in clinical trials. The failure rate was highest in Phase II studies, and lack of efficacy was one of the principal reasons for termination [1]. There is therefore an urgent need for methods to screen potential drug targets and potential toxicity effects early in the development process, so that pharmaceutical companies can choose the best targets for further pre-clinical and clinical development.

To this end, ATERO-EXPRESS (Figure 1) provides a unique biobank and large data repository that:

- Will reveal associations of potential drug targets with patient characteristics, histological and genomic data and in the future, epigenetic, transcriptomic, proteomic and immunological data;
- Uses cutting edge techniques for early screening of vascular effects by drug targets;
- Will be continuously updated with deep phenotyping data;
- Will fuel drug development and increase the chance of potential drugs to reach the clinic and eventually the market.

Despite a significant decrease in mortality over the last decades, cardiovascular disease (CVD) remains the leading cause of death in the United States and the rest of the world. By 2030, heart disease and stroke will result in an estimated 24 million deaths per year worldwide, and thus will continue to be the dominant cause of death. Therefore, the burden of cardiovascular disease remains a major public health concern and a growing global challenge [2]. Unwanted side effects but also beneficial effects on human vasculature is a key milestone in the research and development program of any drug target.

c. Mission and vision

Mission

ATHERO-EXPRESS strives to accelerate cardiovascular drug development and to reduce late-stage failure of drug candidates by enabling early screening of potential drug targets in a human atherosclerosis biorepository.

Vision

ATHERO-EXPRESS will increase the availability of effective CVD treatments and help to reduce the burden of CVD by early screening of potential drug targets.

Potential Cardiovascular Drug Targets

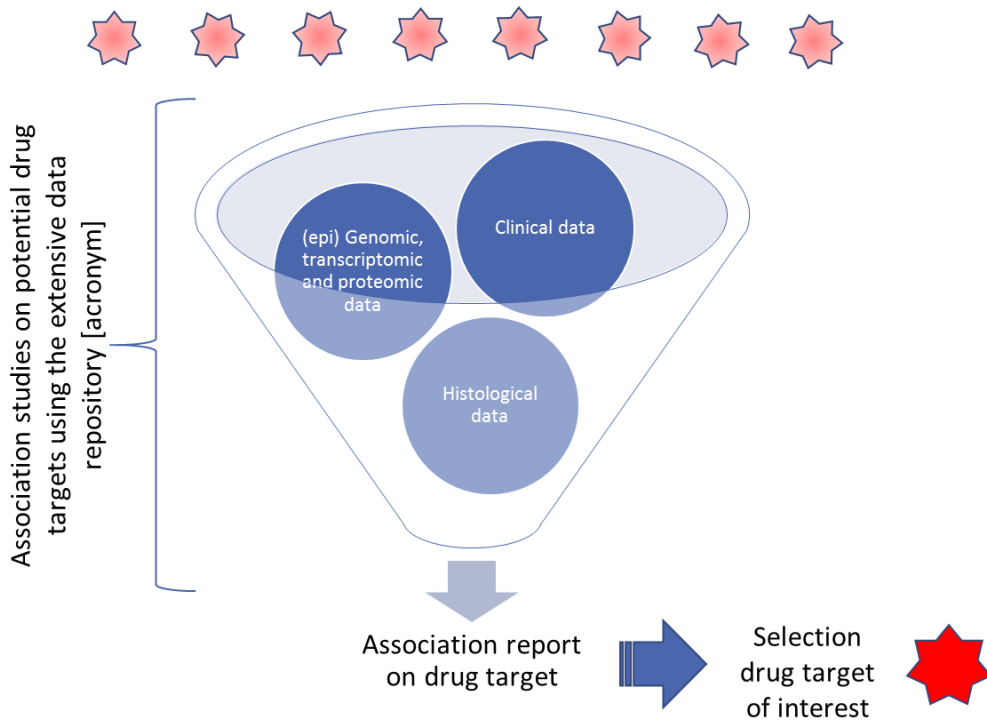


Figure 1: Schematic overview of Athero-Express.

Management

a. Management team

The management team combines complementary expertise on cardiovascular diseases, biobanking, genetics, transcriptomics and bioinformatical analyses to ensure successful exploitation of ATHERO-EXPRESS for improvement of the understanding the effects of gene/protein downregulation in vascular tissue.



The University Medical Center Utrecht (UMC Utrecht) is one of the largest academic healthcare institutions in the Netherlands. The research of the Pasterkamp lab focuses on unravelling the mechanisms of arterial occlusive disease (atherosclerosis) and the repair of vascular tissue.

In 2002 the Athero-Express atherosclerotic tissue bank was established by the department, as a dedicated tool for genome-wide association studies. During the last decades, the Athero-Express biobank has been used 1) to study the predictive value of locally expressed proteins for adverse cardiovascular events in patients undergoing endarterectomy; 2) to study differential expression in atherosclerotic plaques and/or blood cells among patient groups and plaque phenotypes; and 3) to identify subgroups of patients and atherosclerotic plaque characteristics with a high risk for adverse cardiovascular events by combining genetic (whole genome SNP analyses), proteomic and clinical data. The Athero-Express researchers also initiated an extended database, including proteome, transcriptome and epigenetic data, and a second vascular biobank study with a longitudinal study design, the basis for ATHERO-EXPRESS. Within the ATHERO-EXPRESS consortium there is thus extensive experience in setting up, sustaining and analysing large human biorepositories. Moreover, the Athero-Express biorepository is fully at the disposal of the ATHERO-EXPRESS initiative, providing the opportunity to find associations between potential drug targets, expression and disease phenotypes.



Prof. dr. Gerard Pasterkamp is professor at the department of Experimental Cardiology at the University Medical Center Utrecht (UMCU), head of the research group Experimental Cardiology and the research Laboratory of Clinical Chemistry. Prof. Pasterkamp's research focuses on biomarkers associated with cardiovascular disease, biobanking (Athero-Express, Circulating Cells), and CVD-related genetic alterations affecting DNA, RNA and protein processing and modification.



Dr. Hester den Ruijter is a clinical epidemiologist, with extensive experience in coordinating inter-academic consortia, such as the Dutch Heart Foundation consortium Queen of Hearts. Her current research focuses on discovery of sex-specific (female) biomarkers for cardiovascular disease.



Dr. Sander W. van der Laan, is a medical biologist with extensive experience in the genetics and genomics of atherosclerotic disease. His work currently focuses on integrating fine-mapping, co-localization, causal inference quantitative trait mapping, and computer vision of histological slides to identify causal genes amenable to therapy.



Michal Mokry, MSc is a molecular geneticist with expertise in DNA and RNA sequencing in the field of cardiac and vascular diseases. He is in charge of the epigenetics facility in the research group that also analyses the data.

Business Idea and Technology

a. Introduction

The Athero-Express data repository and ongoing analytical platform is applied for: A- general screening of parallel effects on gene and protein expressions that associates with expression levels of any specified drug target. This could point to potential side effects. Specifically the single cell sequencing data could address these potential interactions in specific cell types (such as endothelium). B- reveal the potential effect of cardiovascular drug targets on atherosclerotic plaque characteristics and vascular tissue and cell specific gene expression levels.

Hurdles in drug development

In order to manage a large unmet medical need, pharmaceutical companies are continuously investing in drug development programs. However, there is a high failure rate of drug trials, discouraging innovative drug development. The latter is specifically applicable to cardiovascular drug development programs. For example, development of cardiovascular drug candidate evacetrapib was discontinued by Eli Lilly due to insufficient efficacy in a large-scale Phase III trial. Evacetrapib is a cholesterylester transfer protein (CETP) inhibitor, designed to increase levels of high-density lipoprotein cholesterol - which was hypothesized to provide a protective cardiovascular effect. However, the 12,000 patient ACCELERATE study showed that evacetrapib did not reduce cardiovascular events. Moreover, a significant increase in hypertension was shown in patients taking evacetrapib. Similar drugs developed by Roche (dalcetrapib) and Pfizer (torcetrapib) have also failed at a late development stage due to safety issues emerging in larger clinical trials. The cost of the failed evacetrapib trial amounts to an estimated \$90 M, the cost to Pfizer for discontinuing the ILLUMINATE trial for torcetrapib was reported to be \$800 M [3-5].

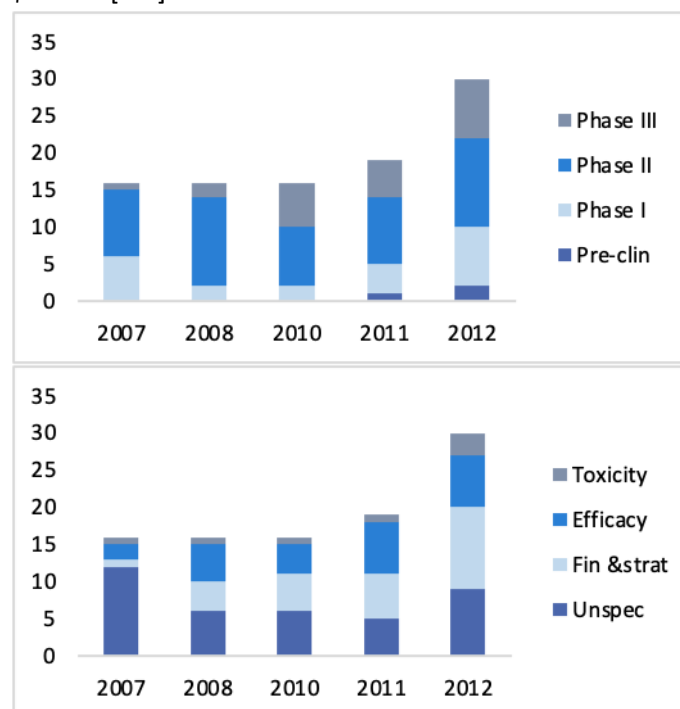


Figure 2: Discontinued cardiovascular drugs 2007, 2008, 2010, 2011 and 2012 by phase (left) and reason for termination (right) (Adapted from [3]).

Analysis of discontinued drug candidate programs in recent years has shown that a relatively large number of drug trials is terminated in the later development phases, predominantly in Phase II. In addition, although financial and strategic decisions are also a factor, up to a third of all drug trials is terminated due to toxicity or efficacy issues (Figure 2).

A more profound screening of potential drug targets based on human data is needed to avoid such drug development failures in the future. This can be done by studying the expression of potential drug targets in relation to genetic variants, to clinical presentation and deeply phenotyped plaques in human biobanks. In this

manner, mechanistic insight into the expression, role and function of the potential drug target will be gained supporting the selection of promising drug targets.

CVD and atherosclerosis

CVD is the leading cause of mortality worldwide. An underlying cause of the majority of CVD cases is atherosclerosis, which is defined as a chronic inflammatory disease of the arterial wall marked by the formation of atherosclerotic plaques. The pathogenesis of atherosclerosis is complex, with a key role for immune cell activation and inflammatory mediators in conjunction with hyperlipidaemia [6]. Depending on the location of major plaques, atherosclerosis may lead to different forms of CVD, such as coronary heart disease, stroke, or carotid and femoral artery stenosis. Statins, which potently decrease LDL-C levels and reduce cardiovascular morbidity and mortality, are currently the first-line drugs for treatment of dyslipidaemia, but these do not address the entire spectrum of CVD risks. Indeed, the residual risk of major vascular events among patients with coronary heart disease remains >20% over 5 years, even with statin treatment [7]. Thus a medical need remains for effective treatment of atherosclerosis, which is the cause of 40% of all cardiovascular deaths [8].

b. Technology

To provide the urgently needed data for drug development programmes, pharmaceutical companies need to access relevant and sufficiently large repositories of patient samples and data. ATHERO-EXPRESS currently houses a data resource that allows powerful association studies, as shown in Table 1:

The current database already provides the ATHERO-EXPRESS consortium with a very strong tool to analyse associations between plaque phenotype and genetic variants of drug targets. At present RNA sequencing is performed for 700 plaques and inclusion for single plaque cell RNA sequencing is ongoing. To extend the database, ATHERO-EXPRESS has planned expansion of genotyping of the samples and further analyses to achieve epigenetic characterisation of all plaques, proteome and transcriptome characterisation of carotid lesions, a cytokine and chemokine profile of all plaques. Additional geno- and phenotyping studies can be planned in consultation between the core consortium and private partners, depending on proposed drug targets and technological opportunities.

Patient characteristics
<ul style="list-style-type: none"> • A patient cohort with high diabetes prevalence. • Detailed baseline and follow-up clinical characteristics of patients.
Tissues:
<ul style="list-style-type: none"> • Plaque biobanks with ~2,400 carotid, ~1,100 femoral and 650 aneurysm lesions.
Plaque characterisation:
<ul style="list-style-type: none"> • Histological phenotyping of all atherosclerotic plaque lesions
Genome-wide genotypes:
<ul style="list-style-type: none"> • Genome-wide genotype data of ~2,200 carotid, and ~400 aneurysm samples. • RNA sequencing data of 700 plaques • Single cell plaque RNA sequencing data (currently 50 patients and inclusion ongoing)
Plaque epigenetic data:
<ul style="list-style-type: none"> • Genome-wide DNA methylation data of ~700 carotid plaque samples.
Plaque immunological data:
<ul style="list-style-type: none"> • Data on >40 immunologically relevant proteins in ~450 carotid lesions. OLINK analyses scheduled for 200 lesions
Data on other relevant cells and tissues:
<ul style="list-style-type: none"> • Genome-wide SNP data, whole-genome expression data of monocytes and stored cell fractions of ~500 patients undergoing coronary catheterization.

Table 1: Current ATHERO-EXPRESS data repository (a detailed description of tissues and data can be found in Appendix 1).

c. Unique selling points

- ~4000 patient samples with complete accompanying molecular data, including genomics, phenotyping and epigenetics.
- In-house expertise to perform analyses to further expand the database.
- Technical and disease-specific expertise for generation of on-demand reports.
- No claims on foreground knowledge or IP.

d. Project examples

The ATHERO-EXPRESS projects can be performed in collaboration with a private partner for verification and (cell-specific) gene-gene associations of a potential drug target. To provide an overview of the possibilities of ATHERO-EXPRESS, we here present four examples based on currently available data. These include:

- 1) Association analysis of a variant at a locus (7q22) that has not yet been considered as a drug target. This study shows atherosclerosis-specific associations between the variant, phenotypic plaque characteristics and gene expression in other tissues.
- 2) Association of expression of a failed drug target (COX-2) in single cells and how these expression levels associate with expression of genes in all vascular cell types. These type of analyses could have pointed to expected downstream (side) effects of a drug that downregulates COX-2.
- 3) Association analysis of a variant of a gene that is currently a widely studied drug target (PCSK9). This study shows novel associations between the variant and plaque characteristics, pointing to additional mechanisms through which inhibition of this drug target might affect atherosclerotic plaques.
- 4) The use of vascular tissue biobanks to unravel epigenetic effects. This study shows associations between epigenetic changes and environmental risk factors for atherosclerosis.

These associations are based on currently available histological data and genome-wide SNP data. In ATHERO-EXPRESS, further deep phenotyping studies will unravel more associations with RNA and proteome based expression levels, as well as with interleukin, chemokine and protease levels. In addition, analyses of genome-wide epigenetic modifications in association with differential plaque characteristics will add to the body of knowledge.

1. Association analysis of locus on 7q22

This locus on chromosome 7q22 has not yet been considered as a drug target, although the variant (rs12539895) on chromosome 7q22 has previously been associated with CAD [9, 10]. In the Athero-express biobank, further associations were found with the carotid plaque phenotype. Both the coronary artery disease risk-reducing A-allele of rs12539895 ($p < 5.1 \times 10^{-6}$, Figure 4) and a nearby deletion (chr7:106,901,393 TG > T) at 7q22 were found to be associated with less fatty carotid plaques (Figure 5).

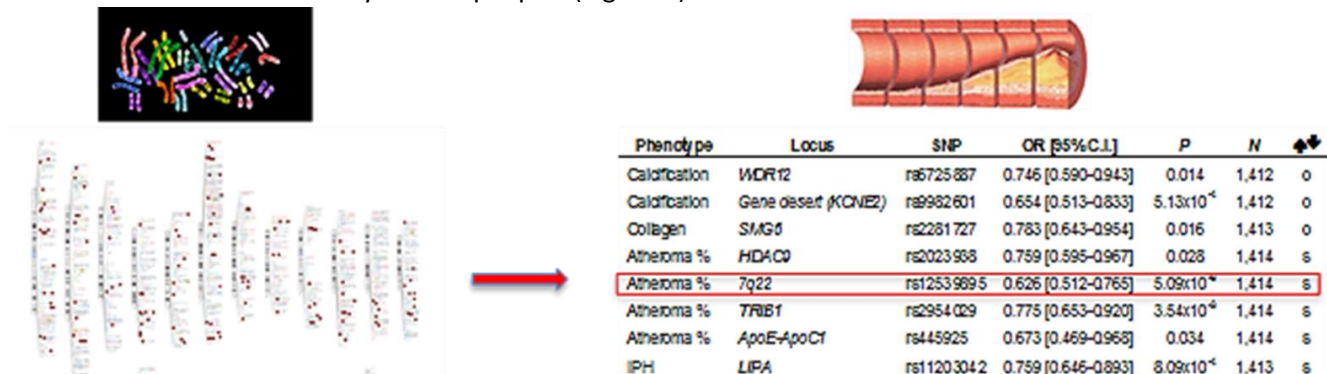


Figure 4: 61 variants related to coronary artery disease were associated with stable and vulnerable histological plaque characteristics. The table shows 8 examples out of 21 associations with plaque characteristics found in the Athero-Express

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study. An example is provided that reveals an association between the SNP in 7q22 that strongly associates with fat content in plaques.

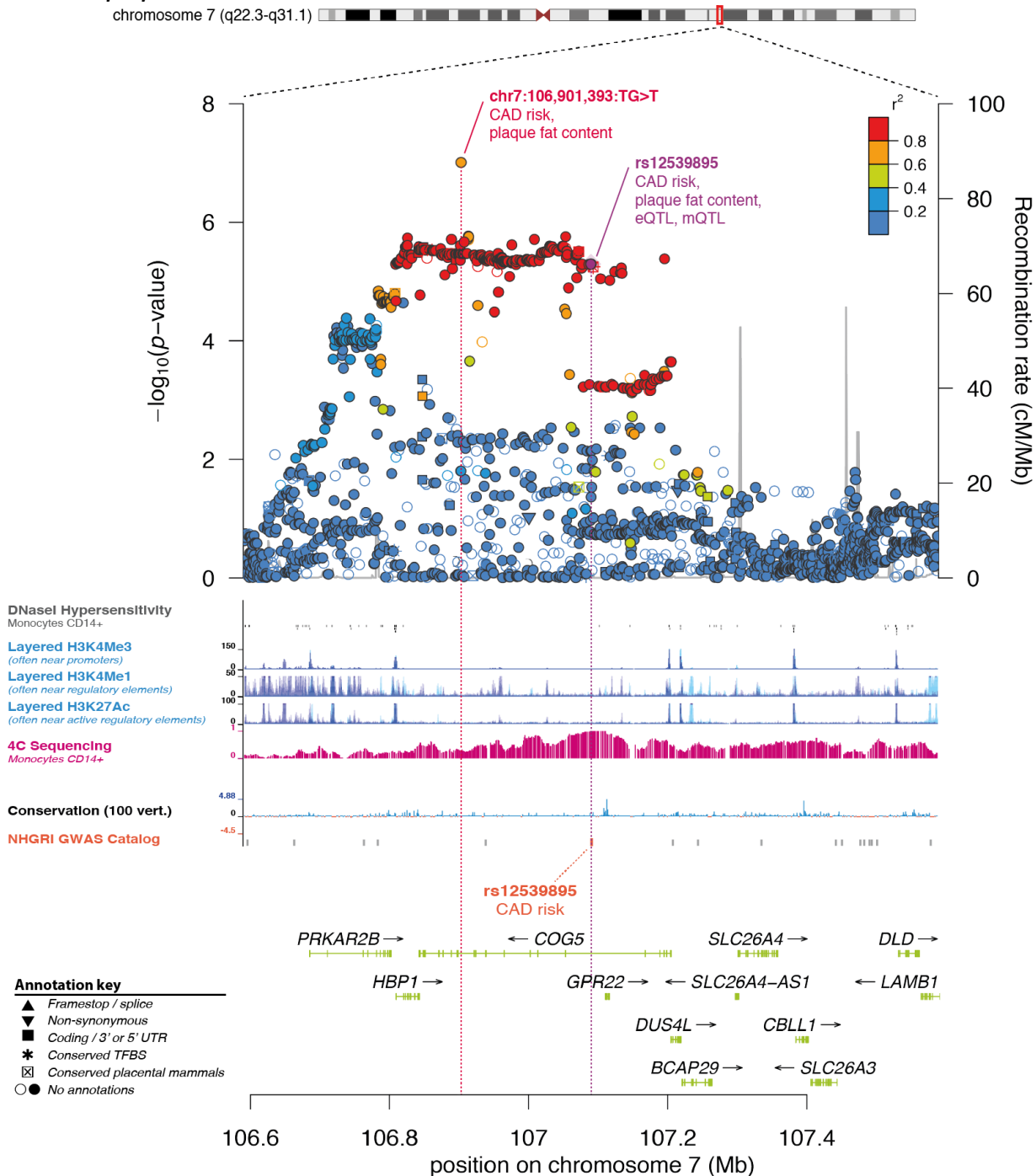


Figure 5: Manhattan plot of the 7Q22 locus depicting the association with lipid content in carotid plaques. Multiple SNPs associate with lipid core size in the atherosclerotic lesions. Subsequent analyses showed an association of the SNP with lipid blood profiles. These data clearly suggest a role of the causal gene in lipid metabolism and supports the view that geno-phenotypic associations in human lesions facilitates decision making on drug target selection.

Further analyses revealed that the genetic variant on chromosome 7q22 was also associated with expression levels of nearby genes in different tissues. Specifically, the risk-reducing A-allele of rs12539895 was associated with reduced COG5 expression in whole-blood cells, atherosclerotic arterial wall, and internal mammary artery tissue. Conversely, the same allele was associated with increased HBP1 expression in carotid plaques and liver cells.

The association of the variant with liver gene expression suggested a causal relation with lipid metabolism, in which the liver plays a central role. Indeed, we found a significant association of the allele variant with lower levels of LDL-cholesterol and higher levels of HDL-cholesterol in blood. Considering the association of rs12539895

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with less fatty carotid plaques, as described above, and the central role of the LDL receptor in lipid uptake, regional gene expression in carotid plaques may also be correlated to LDL receptor expression. This was confirmed in this study by significant associations of LDL receptor expression with 4 genes located near the 7q22 locus (PRKAR2B, COG5, DUS4L and CBLL1).

2. Single cell RNA sequencing of atherosclerotic plaques

We are currently successfully executing single cell sequencing of atherosclerotic plaques. Procedures have been performed in 30 patients and inclusion is still ongoing. These studies provide unique opportunities. We are able to assess cell specific expression of genes of interest (Figure 6). Data can be used to assess cell specific associations of genes of interest with other gene networks. This can provide insight how expression profiles differ in the presence or absence of a gene expression of interest. The latter can be applied to assess wanted pleiotropic or unwanted side effects of affecting any drug target. Examples for COX-2 are provided (Figures 7-9)

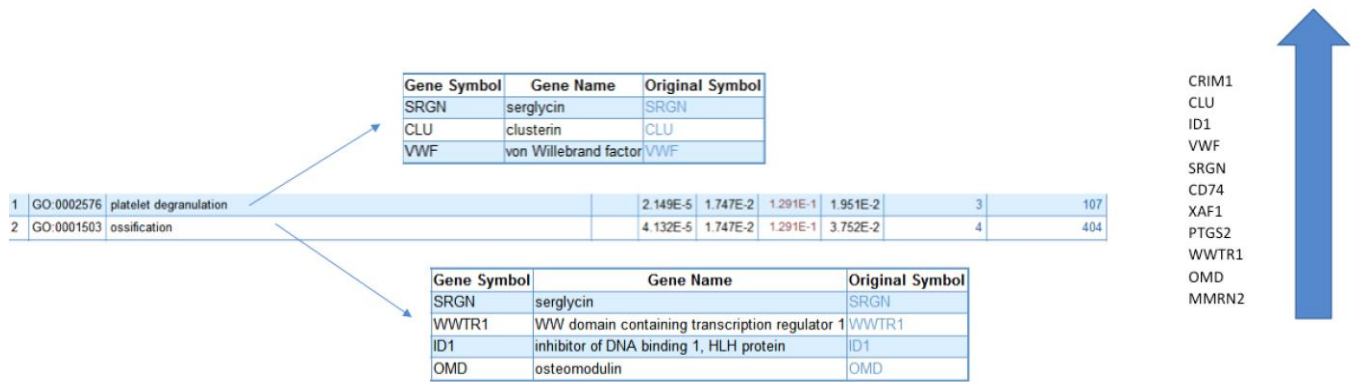
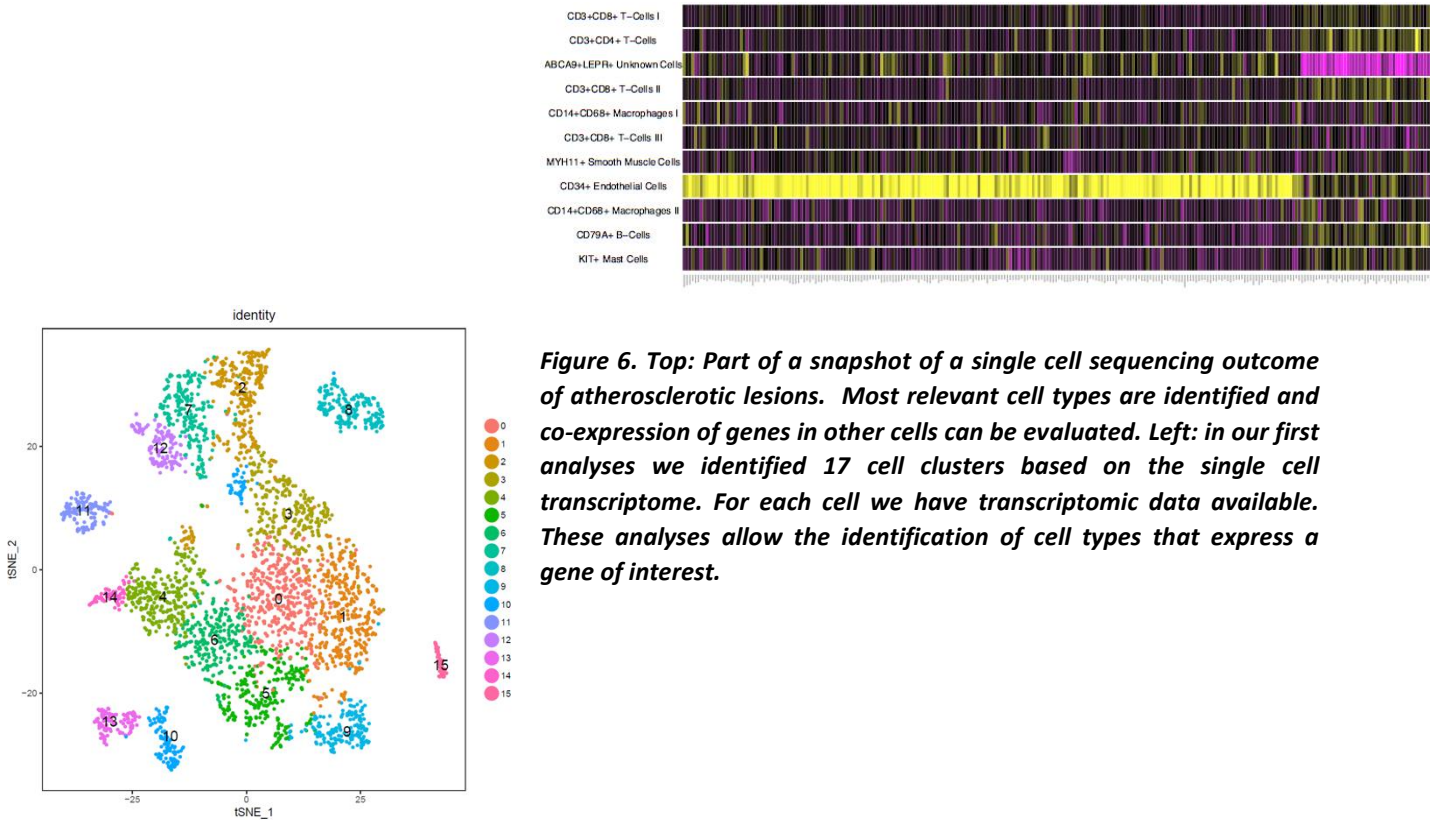


Figure 7: Positive associations of COX-2 expression with other genes in plaque derived lymphocytes

ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
MP:0002419	abnormal innate immunity		1.289E-7	1.261E-4	9.718E-4	1.613E-4	8	627
MP:0003009	abnormal cytokine secretion		2.015E-7	1.261E-4	9.718E-4	2.521E-4	8	664
MP:0002406	increased susceptibility to infection		3.027E-7	1.262E-4	9.732E-4	3.787E-4	7	447
MP:0002723	abnormal immune serum protein physiology		5.155E-7	1.477E-4	1.139E-3	6.449E-4	9	1092
MP:0008568	abnormal interleukin secretion		5.905E-7	1.477E-4	1.139E-3	7.387E-4	7	493

FCN1
MS4A6A
LAPTM5
MARCKS
PTGS2
GNAI2
TYROBP
GRN
COX4I1
SERPINA1
IER3
EGR1
LYZ
CTSS
IL1B



Figure 8: Positive associations of COX-2 expression with genes in plaque derived endothelial cells and fibroblasts

1	GO:0048407	platelet-derived growth factor binding	7.366E-8	6.040E-6	3.014E-5	6.040E-6	3	12
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Gene Symbol	Gene Name	Original Symbol
COL3A1	collagen type III alpha 1 chain	COL3A1
COL6A1	collagen type VI alpha 1 chain	COL6A1
COL1A2	collagen type I alpha 2 chain	COL1A2

S100A4
GAPDH
LGALS1
MYH9
COL3A1
THBS2
CFH
ACTA2
MYH10
TAGLN
LPP
COL6A1
COL1A2
MYL9



Figure 9 : Negative associations of COX-2 expression with genes in plaque derived endothelial cells and fibroblasts

3. Association analysis of PCSK9

PCSK9 is a drug target which is currently investigated by the pharmaceutical industry, and several Phase III studies to study the effect of PCSK9 inhibition on clinical CAD-related events are ongoing. PCSK9 binds to the LDL-receptor, inducing its degradation and decreasing LDL-C metabolism.

We studied the association of genetic variants in the *PCSK9* gene, which are known to be associated with coronary artery disease, with human histological atherosclerotic carotid plaque characteristics. We found that the *PCSK9* variant (rs505151: E670G) associated with one of the stable plaque characteristics, namely collagen content (Table 2, right). We further replicated the association of rs505151 with plasma levels of PCSK9 in these patients (Table 2, left). Lower blood levels of PCSK9 were associated with increased collagen content in the atherosclerotic plaques. Current research is exploring whether this association is a direct causal effect of PCSK9 on collagen turnover or an indirect effect via the known effects of PCSK9 on LDL reduction. The first *in vitro* results point to a direct effect of *PCSK9* on collagen turnover.

Table 2: Associations between low plasma levels of PCSK9, rs505151 (E670G) and various plaque characteristics in the Athero-Express Biobank Study.

Plaque phenotype	ATHERO-EXPRESS Plasma PCSK9		SNP rs505151	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Presence of IPH	0.93 (0.81-1.07)	0.31	0.89(0.49-1.64)	0.7
Presence of Collagen	1.32 (1.10-1.59)	0.003	2.84 (1.14- 9.5)	0.048
Presence of macrophages	0.95 (0.83-1.09)	0.45	1.34 (0.72-2.55)	0.36
>10% fat content	0.97 (0.84-1.13)	0.69	1.58(0.79-3.5)	0.22
Presence of SMC	1.09 (0.93-1.27)	0.29	1.35 0.70-2.80)	0.4
Presence of calcium	0.95 (0.82-1.09)	0.44	0.79 (0.42-1.43)	0.43
Thrombus percentage	1.89 (0.54- 6.55)	0.32	0.92 (0.01- 70.95)	0.97
Average vessel density	1.56 (0.98-2.49)	0.06	0.66 (0.243-1.81)	0.42

4. Risk factors, medication use and the effect on the atherosclerotic lesion epigenome

While common DNA variation is inherited, epigenetic changes of DNA can be induced by environmental changes such as diet or medication. Both genetic and epigenetic variations may carry a similar effect on gene transcription. Extensive data on environmental risk factors and medication use are available in the ATHERO-EXPRESS data repository and can be associated with significant changes in the epigenome of atherosclerotic lesions. The assessment of epigenetic modifications will serve multiple purposes:

1. Epigenetic changes in a proposed drug target will influence expression and phenotypic changes. These changes can be projected on biological pathways and hence can serve as surrogate measure of efficacy.
2. Risk factors, such as hypercholesterolemia or smoking, induce epigenetic changes and subsequent gene expression. Target discovery programs will benefit from this epigenome-phenotype linkage studies.
3. Gene methylation could serve as a proxy for drug effects. Studying epigenome-phenotypic associations is an experiment by nature to search for potential effects of drug targets.

A risk factor, for instance smoking, influences gene expression in vascular tissue. In an epigenome-wide association study (EWAS) to investigate the association between tobacco smoking and DNA methylation in atherosclerotic plaques from carotid arteries, 68 CpGs (methylation sites) were associated with current tobacco smoking (Figure 10). Of these CpGs, 12 were previously associated with smoking in a study on blood cells and 40 CpGs were novel loci. The strongest association was observed for CpGs in the gene *AHRR*. This is a repressor of the aryl hydrocarbon receptor transcription factor, which is involved in xenobiotic detoxification, and was previously been identified in blood cells. Six of the novel CpGs were associated with major cardiovascular events within three years of follow-up.

In addition to risk factors, use of medication also influences epigenetic modifications. Assessment of plaque methylation loci that associated with statin use yielded one genome-wide hit on chromosome 18 (Figure 11).

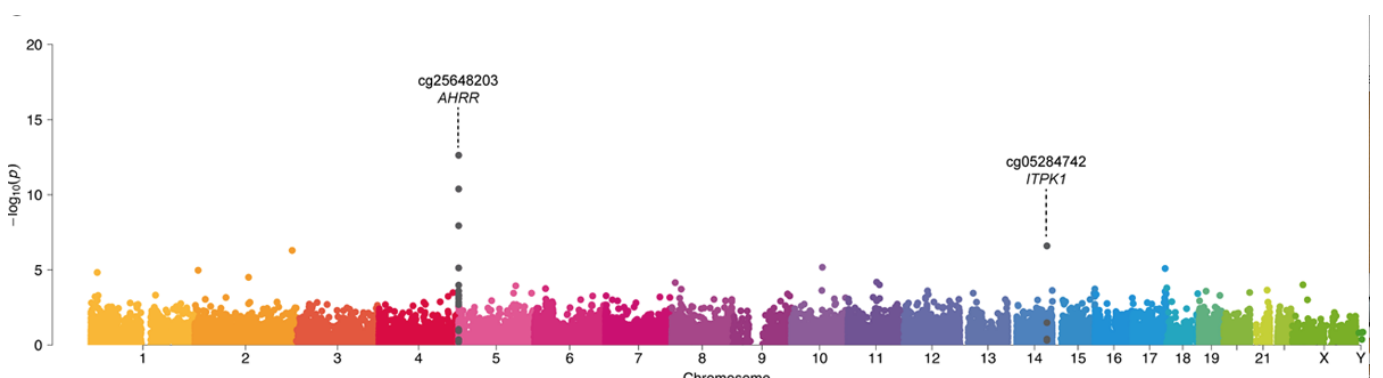


Figure 10: Atherosclerotic plaque methylation sites in association with current tobacco smoking. DNA methylation of specific genes takes place in vascular tissues which will affect gene expression. Subsequently, the association of methylation of specific genes will be associated with plaque and clinical characteristics. These associations further support a causal role in disease progression of any gene of interest. These associations can also be unravelled stratified for specific medication use (see next figure)

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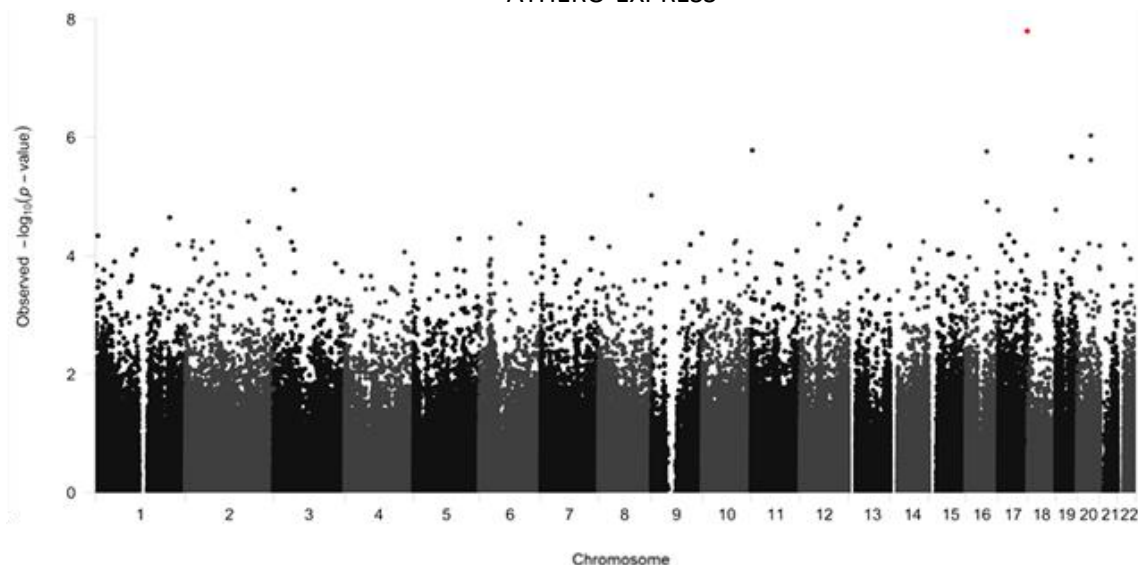
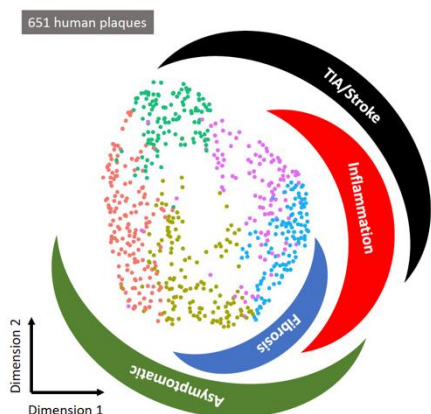


Figure 11: Atherosclerotic plaque methylation sites (in red) in association with statin use. We compared methylation of genes in atherosclerotic plaques (which will subsequently affect expression) in association with statin use. In this example we find a genome wide hit in a gene in chromosome 18.

Novel description of the “vulnerable plaque” that elicits clinical events by merging pathology and transcriptome data.

The classical concept of the ‘vulnerable plaque’ that depicts the plaque rupture as the major pathological substrate for acute cardiovascular events originated in the 70ties and 80ties from observations in patients who succumbed to fatal acute coronary syndromes. This recognition spawned a generation of research that led to greater understanding of how complicated atherosclerotic plaques form and precipitate into thrombotic events. Current evidence suggests that a sole focus on plaque rupture of atheromatous lesions has vastly oversimplified the complex collection of atherosclerotic diseases and obscured other mechanisms that may mandate different management strategies.

The mechanisms that underlie superficial erosion, a cause of coronary thrombosis distinct from plaque rupture, have garnered increasing interest. In an era of improved control of traditional risk factors, plaque erosion assumes greater clinical importance. In addition, the widely available analyses of genomic variants expressions profiles revealed novel and unexplored causal genes that associate with the occurrence of acute atherosclerotic cardiovascular events. The field of human genetics exposed many loci that with unknown functions. About 45% of the known CAD risk loci are all cell-specific and map to known genes involved in lipid metabolism, blood pressure, and glucose homeostasis. However, for the majority of risk loci the underlying gene regulatory and cell specific networks have yet to be clarified.



At present the pathological definition of the plaque at risk for acceleration towards a clinical event is dominating clinical and animal research. The increasing availability of bulk gene expression data in human atherosclerotic plaques will result in the description of tissue gene clusters that show consistent associations with clinical outcome. We hypothesize that a combined gene and pathology based description of the dangerous atherosclerotic plaque will reveal an improved association with clinical events than the pathological description only. In addition, we assess if relevant gene clusters that could not be optimally discriminated by pathology associate with biological processes and expression in specific celltypes. This

study is ongoing but first results provide evidence that the plaque transcriptome significantly contributes to a definition of the plaque that associated with clinical events on top of the pathological characterisation.

Figure 12 (previous page) . Clustering of atherosclerotic plaques based on bulk RNA sequencing. 5 patient clusters are identified that associate with pathology and with clinical symptoms.

Exploitation strategy for ATHERO-EXPRESS

a. Development strategy

The ATHERO-EXPRESS team aims to exploit and enrich their existing, unique database on vascular diseased tissues and, using the database, provide association data on drug targets that are of interest to the pharma industry. The consortium will perform analyses of the biobank samples to achieve deep phenotyping of these samples, adding data on genetic variants, proteomics and immunology. In addition, we will provide atherosclerotic plaque **cell specific** associations of expression levels of genes of interest. The ATHERO-EXPRESS core team will perform (in collaboration) each of the analyses and association studies, making optimal use of our joint specific expertises. If desired by the private partner, additional analyses can be performed, focused on specific potential targets such as, protein expression and immunohistochemical localisation.

The private partner will be offered the opportunity to propose potential drug targets for screening within the database. In collaboration with the partner we will perform the screening on the cell specific and atherosclerotic lesion-specific associations of the gene of interest, in return for a fee to be used for further development of ATHERO-EXPRESS.

The full information package that will become available during the project will include data on:

1. Associations of human (epi)genetic variants and human plaque phenotypes of proposed drug targets.
2. Lesion cell specific associations of expression of genes of interest with gene networks.
3. Associations of human (epi)genetic variants and human genome-wide atherosclerotic plaque and circulating cell transcriptomic and proteomic expression of proposed drug target genes.
4. Associations of genetic variants, gene and protein expression in cells and plaques with clinical presentation and follow up.
5. Verification of outcomes from 1. and 2. in large human GWAS studies on coronary artery disease.

b. Knowledge management & Intellectual Property

Confidentiality and publications

The ATHERO-EXPRESS database comprises data that originate from public bodies, i.e. the biobanks established by core consortium partner UMC Utrecht. Scientists will address research questions using these data, and may therefore disclose data from the database, which are of interest to a private partner, to the public domain.

When a private partner confidentially shares one or more target(s) of interest, the potential for a conflict of interest will be assessed and, if present, disclosed to the private partner. When the proposed targets do not appear in the interest domain of researchers at the ATHERO-EXPRESS, then data will not be disclosed to the public domain for at least 1 year.

The right of private partners to halt publication of results for a period of time if conflict of interest arises, will be specified in the consortium agreement, but will be at least 60 days.

Ownership of data

Each partner (public or private) will retain all rights to their privately owned material and information. No right or license to the data is granted by any partner either expressly or by implication, except those specifically set forth in the consortium agreement. All rights, trademarks and proprietary rights to the material and information, shall remain with the partners, and are subject to the rights defined in the consortium agreement. No restrictions on

the academic partners' right to the material will apply, except that is specifically set forth in consortium agreement.

Intellectual property

The ATHERO-EXPRESS team will not make claims on foreground knowledge and does not intend to deliver on the basis of royalty agreements in drug target screening studies requested by any private partner. If a partner proposes a joint drug discovery program in addition to the drug screening studies, a model for intellectual property rights will be discussed in good faith. A format for this model will be part of the consortium agreement.

Exclusivity of targets

ATHERO-EXPRESS is a pre-competitive initiative: all data are provided in confidentiality to each partner, but on a non-exclusive basis.

c. Quality strategy

Biobanking

All samples included in the ATHERO-EXPRESS repository have been collected in dedicated biobanks at the UMC Utrecht (Athero-Express, initiated in 2002). The histological analyses in Athero Express biobank have undergone extensive intra- and interobserver analyses. In addition, an automatic quantification platform for histology materials has been developed and is fully operational [11]. All +/- 25.000 histological slides have undergone this fully automated analysis. The samples will remain under the control of the institute.

Analyses

Each of the planned sample analyses will be performed according to each partner's specific expertise. Standard operating procedures will be drafted for each method, including procedures for central and secure deposition of analysis results in the central data repository.

Data access & reports

All data in the repository are anonymized, and access to the databank will be restricted to the consortium researchers. The consortium will take into account all applicable EU and US directives concerning data storage and exploitation.

Data reports will be compiled by the central front-office, according to a standardized format. ATHERO-EXPRESS will make use of a documentation system including biobanking, laboratory data, clinical data and reports so as to create an infrastructure for documenting its own database development and for executing studies for the private partners. Such a documentation system captures the valuable know-how of all developmental work in ATHERO-EXPRESS.

Ethics and consent

All tissues and data in the biobanks originate from patients who have given informed consent according to the procedures at the respective institutes. Patients have provided explicit consent for use of their tissue and data for research purposes (secondary use).

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Table 3: Realization steps in ATERO-EXPRESS consortium

Steps	Description	Status
Histological analysis of 2,400 carotid, 1,100 femoral, 4,696 coronary and 650 aneurysm lesions (completed)	The histological phenotypes of all available plaque samples have been analysed.	Milestone reached.
Genome-wide genotyping of 2,400 carotid and 400 aneurysm samples (completed)	The genotypes of carotid and aneurysm patients have been determined.	Milestone reached
DNA, RNA and protein isolations (completed)	RNA is currently undergoing sequencing for 700 lesions	Milestone reached.
Harmonization of histological analyses (completed)	All histological sections are scanned and automated analysis performed.	Milestone reached.
Whole-genome methylation analyses of all plaques (completed)	Whole-genome methylation analyses for 700 carotid plaque samples are available.	Milestone reached
Whole-transcriptome analyses of all carotid atherosclerotic lesions (completed)	Samples of 2,400 carotid plaques are available, we have finished sequencing of 700 lesions in 2019.	Milestone reached
Whole proteome analyses of all carotid atherosclerotic lesions (planned)	Samples of 2,400 carotid plaques are available, matrix protein proteomics planned in 2019-2020 of 300 lesions	Milestone planned in 2020.
Analyses of cytokines and chemokines of 500 available plaques (planned)	Expression of cytokines and chemokines are available in 700 lesions and in 2020 500 additional plaques will be measured on a validated commercially available platform (i.e. Olink based).	Milestone partly reached and pending 2020.
Single cell sequencing of plaques of carotid and femoral artery lesions (partly completed)	We have included 50 plaques and will continue. We propose to continue including beyond n=50.	Milestone partly reached but extension pending on collaborations

d. Risk and contingency planning

Since the current data-repository offered by ATERO-EXPRESS already offers extensive screening opportunities, risks associated with investing in this consortium are limited. The core team has all expertise in-house to perform the required analyses within the project and we are open for collaborations to study data in depth. This will provide the consortium with an expanded database, from which the experienced core investigators will derive association data on drug targets of interest.

Scientific drivers

In conjunction with the industrial needs, as described above, there is now great momentum from a scientific viewpoint to use human biobanks such as applied in Athero-Express in early drug target screening for the following reasons:

Exploiting the GWAS-guided genetic approach for candidate genes

Genome-wide association studies (GWAS) have revealed thousands of genetic variations associated with diseases. For example, in coronary artery disease and stroke >150 associated loci have been discovered, and as sample sizes increase, it is expected that more loci will be discovered in the near future. Despite initial promising results however, thus far only few drug targets have been found based on GWAS, also because these have predominantly identified non-coding sequences. However, these non-coding sequences are largely in key regulatory regions of the genome. Therefore, the target genes of these regulatory elements can also be considered candidate genes. Use of these biological principles yields an alternative method to identify and screen candidate genes for human diseases. Circular chromosome conformation capture followed by sequencing (4C-seq) so far has identified novel candidate genes for atherosclerosis [15].

Exploiting the causality of candidate genes

A causal role for any proposed target of interest can be assessed using Mendelian randomization studies, supporting decision making in drug development. A retrospective study has demonstrated that many drugs that are successfully applied in the clinic would have been supported by strong geno-phenotypic associations with the targeted disease during development. This was shown by a post hoc assessment of Phase III clinical trial successes and failures, which found that all targets for which clear genetic evidence was available and good pharmacological agents were developed, produced the clinical effect that was predicted by the genetic studies [16]. A lack of geno-phenotypic association may also predict clinical failure, as studies using Mendelian randomization have now shown that increased HDL-C cannot be translated into a reduction in coronary artery disease risk, possibly providing an explanation for the costly failures of CETP inhibitors described above [16].

Replacing animal models for atherosclerotic disease with human (like) models.

Human chronic and life threatening diseases often develop over decades and concepts of disease progression have mainly been based on observational human pathology studies. Animal models have been developed, but these mostly use surrogate measures of disease. A recently published search in literature (>11.000 papers) demonstrated that there is hardly any evidence in human genetic studies for the vast majority of targeted genes that resulted in an atherosclerotic mouse phenotype [17]. Replacement of the early screening phase for potential drug targets in animal models with human data repositories will thus deliver more relevant data.

Competitors/other initiatives cardiovascular biobanks

Consortia and cooperative arrangements, particularly pre-competitive arrangements that generate shared resources such as data, tools and analytics, can help advance drug development. This is an attractive option to make optimal use of the expertise present in academia, and save time and money by generating therapeutics, diagnostics or devices to improve the public health in a more efficient and effective manner.

Several pre-competitive and other public-private consortia have been launched in the cardiovascular field, but to date, no initiative comparable to Athero-Express has been launched in the field of atherosclerosis. The Athero-Express initiative combines a large data repository (~ 9000 samples), with a broad coverage of geno- and phenotyping data, i.e. DNA, RNA and protein levels. In addition, Athero-Express will be focused on improving cardiovascular drug development, by providing extensive screening reports on potential drug targets.

The activities of the main other initiatives in the field are summarized below:

Collaboration AstraZeneca and Montreal Heart Institute

As of May 2015 a collaboration was set up between AstraZeneca and the Montreal Heart Institute (MHI) in Quebec, Canada, to search the genomes of up to 80,000 patients for genes associated with cardiovascular diseases and diabetes, their complications and treatment outcomes. MHI will genotype DNA samples from

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AstraZeneca's extensive biobank using genome-wide SNP analysis to identify regions of DNA that predispose to, or cause, cardiovascular diseases and diabetes or are associated with responses to treatments. No deep phenotyping studies are planned in this collaboration. AstraZeneca will work with MHI to publish findings in peer-reviewed journals, contributing to broader scientific understanding of these disease conditions. No information is in the public domain on financial details of this collaboration.

High Risk Plaque Initiative

The HRP Initiative was initiated in 2006 as a precompetitive industry collaboration funded by BG Medicine, Abbott, AstraZeneca, Merck, Philips, and Takeda, planned to provide a total of \$30 million in funding over four years. This initiative focuses on individuals at risk of atherothrombotic events, who do not display any symptoms. The initiative includes the Bio-Image Study in which novel advanced imaging tools suitable for non-invasive screening for subclinical atherosclerosis are tested in a normal population. The aim is to advance the understanding, recognition, and management of asymptomatic individuals at risk for atherothrombotic events such as myocardial infarction or stroke.

Biomarkers Consortium, Atherosclerosis Modeling Project

The Biomarkers Consortium is a public-private biomedical research partnership managed by the Foundation for the National Institutes of Health to discover, develop, and seek regulatory approval for biomarkers to speed the development of medicines and therapies for detection, prevention, diagnosis and treatment of disease and improve patient care. The Atherosclerosis Modeling Project is a two-year effort to use computer modelling to better understand heart disease and the potential effectiveness of medicines used to treat it. It includes cardiovascular disease experts and computer modellers from leading academic institutions, the National Institutes of Health, Food and Drug Administration, the pharmaceutical and food industry to develop and execute the project. Consortium members providing financial support include Amylin Pharmaceuticals, Eli Lilly and Company, Pfizer Inc., Takeda Global Research & Development Center, Inc., the Dairy Research Institute®, Quintiles and Entelos Holding Company.

Human Blood Plasma Metabolome (HuPMet) Consortium

Under the direction of Professor Christopher Beecher, University of Michigan Center for Translational Pathology, this initiative aims to identify and quantitate all of the small molecule components of human blood plasma that are generally found in > 0.01 nM concentrations (~2000 compounds). In addition, it aims to develop reproducible systems for optimal separation and detection of these molecules and determine the "normal" distribution of each molecule. Industry partners in this consortium include Bristol-Myers Squibb, Pfizer, Takeda Pharmaceuticals, Human Metabolome Technologies and Agilent Technologies.

Collaboration Merck & Co, Inc. And FoxHollow Technologies: atherosclerosis biobank (discontinued)

FoxHollow Technologies, Inc. and Merck & Co., Inc. set up a strategic collaboration in 2006 for atherosclerotic plaque analysis in which Merck acquired a stake in FoxHollow. This included a payment by Merck of \$40 million to FoxHollow over four years in exchange for FoxHollow's agreement to collaborate exclusively with Merck in specified disease areas. Upon extension of the collaboration program beyond this period, Merck would pay \$10 million per year, which may be offset by potential royalty and milestone obligations. The initiative has since been discontinued.

UK Biobank: The UK biobank provides a powerful resource for searches that allow geno-phenotypic associations with any disease. The added value of Athero-Express is the availability of expression levels in advanced human diseased tissues and geno-phenotypic associations in a diseased elderly cohort.

Cost & Funding

a. Financial need

ATHERO-EXPRESS makes use of pre-existent data. However, as part of the project we plan to enrich genotypic and phenotypic data on the large number of plaque samples available. These studies will require investments from grants and private partners. Return on investment is the fast delivery of reports based on readily available data. An estimation of the costs of the studies and operational costs for the consortium for the planned 5 years is provided in Table 4.

Table 4: Estimated costs of ATERO-EXPRESS in future studies.

Athero-Express operational costs (5 years)	
Specification	Costs
Data curation & harmonization <i>Automated analyses of all stains of carotid plaques. Harmonisation of databases & data quality checks.</i>	€ 250,000
Reports and operational costs front office <i>Including consortium meetings, inclusion patients, follow up data, monitoring, travelling, administration, data monitoring, € 300,000 * 5 years.</i>	€ 1.500,000
Laboratory analysis costs (5 years)	
Study type	Costs
Genome-wide genotyping new samples	€ 300,000
Whole-genome methylation analyses <i>On ~1,600 carotid</i>	€ 800,000
Whole proteome analyses <i>On 2,000 carotid samples.</i>	€ 1,200,000
Whole transcriptomic analyses using RNA sequencing <i>On 2,400 carotid samples.</i>	€ 800,000
Protein analyses, i.e. cytokines and chemokines <i>Using antibody or antamer based platforms, upon request partners, on max 2,400 carotid and 1,100 femoral samples.</i>	€ 1,500,000
Single cell RNA sequencing, 200 patients (50/year)	€ 400,000

b. Private investments

Private partners are invited to join this initiative. The subscription rate will be negotiated taking into account the scope of the collaboration. The investments of the partners will be used to improve the operational status of the platform and execute additional analyses, including enrichment of genotypic and phenotypic data.

c. Future funding opportunities

The 5 years objectives of the ATERO-EXPRESS initiative can be fully or partly supported by private partners. However, this does not preclude the possibility of specific spin-off projects being initiated by the consortium, for which public funding, such as R&D grants can be considered. In addition, after successful completion of the studies proposed by the ATERO-EXPRESS consortium, different funding opportunities will be considered to exploit the results of the project. These will be discussed with all partners towards the end of the project, and may consist of both private investments and/or non-dilutive funding opportunities, such as R&D grants.

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Appendix

Currently available data

1. Athero-Express Biobank study

Table 1 Baseline characteristics of Athero-express patients stratified by operation site:
internal carotid artery: n=2344, femoral and iliac artery n=1033

	Carotid artery	Femoral/iliac artery
Number of patients	n=2344	n=1033
Age, mean (SD)	69.1 (9.3)	67.9(9.1)
Gender, male, n(%)	1548 (68.6%)	719 (71.3%)
Body mass index, mean (SD)	26.4(4.0)	26.1(4.1)
Current smoking, n(%)	756(34.2%)	388(39.3%)
Diabetes, n(%)	527(23.3%)	323(32.1%)
Mean arterial pressure, [mmHg].	105.5(17.3)	100.4(14.5)
Hypertension, n(%)	1490(68.8%)	663(69.6%)
Statin use, n(%)	1729(76.8%)	742(74.2%)
Aspirin use, n(%)	848(37.7%)	367(36.7%)
Ascal use, n(%)	999 (44.4%)	428(42.8%)
Oral antiplatelet use, n(%)	1990 (88.6%)	831 (83.1%)
ACE-inhibitors, use n(%)	729 (31.2%)	451 (44.0%)
History of peripheral artery disease, n(%)	480 (21.3%)	981 (97.4%)
History of coronary artery disease, n(%)	734(31.4%)	435(42.35)
Asymptomatic/ ocular symptoms	660 (29.5%)	-
TIA	961 (42.9%)	-
Stroke	617 (27.6%)	-

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Table 2. Questionnaire Athero-Express:

Completed questionnaire from 2280 (97.3%) of patients that underwent Carotid artery endarterectomy and 975(94.4%) from Ileo-femoral endarterectomy patients. Main topic questioned and (n) number of main questions asked. Every main question could have sub questions (e.g 1.1 + 1.1 + 1.3).

Baseline questions patient demographics (15)	Medical history Operation history Other previous medical treatments Comorbidities Employment Weight, length, allergies, hypersensitivity
Family history (30)	First degree family members with history of: hypertension, Diabetes, hypercholesterolemia, pregnancy`s , CVA, CAD, Aneurysms, amputations, thrombosis and other diseases.
Heart (28)	Coronary artery disease including myocardial infarction, coronary interventions, angina pectoris, rhythm disorders, and aortic aneurysms.
Brain (13)	Symptoms experienced; motor/sensor function loss, loss of vision, loss of speech, loss of understanding. History of stroke, TIA, AFX. Symptoms in the past and measures took. Status of coronary arteries(measured bij Doppler ultra sound).
Legs (20)	Detailed information about complaints experiences while exercising, walking or during rest. Location these complaints are experienced (e.g. calf), wound healing problems, previous interventions or therapies.
Risk factors (20)	Various risk factors including hypertension, diabetes and hypercholesterolemia.
Female risk factors (14)	Birth control use, menstruation and pregnancy.
Diet (24)	Detailed information on smoking history, alcohol use (current and history), diet.
Physical activity (10)	Physical activity score based on sports practiced, physical activities performed at work and in and around the house during normal day life.
Quality of life (11)	Validated quality of life questionnaire on experienced quality of life, daily activities, limitations in daily life due to health problems, emotional health, pains experienced, emotional status

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Table 3. Last preoperative measurement, until maximum one month preoperatively. Athtero-express patients stratified by operation site: Internal carotid artery: n=2344, femoral and iliac artery n=1033

	Carotid artery	Femoral/iliac artery
Hemoglobin [mmol/L].	8.7(1.0)	8.4(1.1)
Hematocrit (fraction of erythrocytes in the blood	0.41(0.05)	0.40(0.05)
Creatinin [umol/L].Median (SD)	89.0(46.8)	85.0(83.4)
eGFR based on MDRD formula (mL/min/1.73m ²)	72.6(21.1)	78.1(29.5)
Total cholesterol [mmol/L] (<=5.0 mmol/L) mean (SD)	4.7(1.2)	4.6(1.1)
LDL-cholesterol [mmol/L] (<=3.5 mmol/L). mean (SD)	2.8 (1.1)	2.6 (1.0)
HDL-cholesterol [mmol/L] (male: 0.90-1.70 mmol/L, female: 1.10-2.00 mmol/L). mean (SD)	1.2 (0.4)	1.2 (0.4)
Triglycerides [mmol/L. Median (SD)	1.5(1.1)	1.7(1.5)
Homocysteine [umol/L]. Mean (SD)	13.9(7.7)	14.6(5.8)
Glucose [mmol/L].	6.0(2.1)	6.0(2.4)

Table 4. Athero-express plaque histology measurements, n=total number of patients

	Carotid artery (n)	Femoral/iliac artery (n)
Percentage fat in the plaque (atheroma). Based on HE, EvG and Picrosirius Red staining. [%]	1890	718
Presence of thrombus. Evident from erythrocytes and/or fibrin. Based on HE staining.	1888	720
Presence of calcifications. Based on HE, alfa-actin staining and/or when decalcified remnants are evident.	1889	718
Presence of collagen. Based on Picrosirius Red staining.	1890	718
Presence smooth muscle cells (SMC). Based on alfa-actin staining. [% of plaque area]	1890	718
Presence macrophages. Based on CD68 staining. [% of plaque area]	1890	718
Intraplaque vessels quantified (average number per 3 hotspots).	871	215

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Table 5. Athero-express cytokines and chemokines in plaque measurements.

	Total	Carotid artery (n)	Femoral/iliac artery (n)
Interleuking 6 (IL6; Entrez Gene: 3569)	1237	1219	18
Interleuking 6 receptor (IL6R; Entrez Gene: 3570)	1240	1218	22
Interleukin 8 (a.k.a. CXCL8; Entrez Gene: 3576)	1130	1102	28
Monocyte chemotactic protein 1 (a.k.a. CCL2; Entrez Gene: 6347)	1302	1268	34
SERPINE1 (a.k.a. PAI1; Entrez Gene: 5054)	1303	1269	34
Colony Stimulating Factor 1 (Macrophage) (CSF1, a.k.a. MCSF1; Entrez Gene: 1435)	1304	1270	34
Adiponectin, C1Q And Collagen Domain Containing (ADIPOQ; Entrez Gene: 9370)	1301	1267	34
Cystatin C (CST3) concentration in plaque [pg/ug], measured by Luminex.	874	871	3
Angiopoietin-2 in plaque, measured by ELISA, [pg/ug]	317	312	5
Fatty acid binding protein 4 (FABP4) concentration in plaque [pg/ug]	874	871	3

Table 6. Athero-express proteins in plasma measurements, cutoff point more than 1000 measurements.

	Total	Carotid artery (n)	Femoral/iliac artery (n)
Ip-PLA2 concentration [ng/ml] from citrate plasma	1356	1068	288
PCSK9 in citrate plasma (pg/ml)	1369	1076	293
GDF-15 in citrate plasma (pg/ml)	1369	1076	293
VEGF-A in citrate plasma (pg/ml)	1249	990	259
von Willebrand factor in citrate plasma (pg/ml),	1235	967	268
Osteoprotegerin in citrate plasma [ng/ml]	1438	1116	322
Rantes in citrate plasma [ng/ml]	1458	1131	327
High sensitive CRP in citrate plasma [ug/ml]	1454	1128	326
Thrombin-antithrombin complex in citrate plasma [pmol/L]	1452	1126	326
Myeloperoxidase in citrate plasma [ng/ml]	1458	1131	327
NTproBNP in citrate plasma [pmol/l]	1458	1131	327
PDGF BB in citrate plasma [ng/ml]	1436	1116	320