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Jennifer Frueh

Nataly Maimari

Takayuki Homma

Sandra M. Bovens

Ryan M. Pedrigi

See next page for additional authors

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Authors

Jennifer Frueh, Nataly Maimari, Takayuki Homma, Sandra M. Bovens, Ryan M. Pedrigi, Leila Towhidi, and Rob Krams

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Systems Biology of the Functional and Dysfunctional Endothelium

Jennifer Frueh, Nataly Maimari, Takayuki Homma, Sandra M. Bovens,

Ryan M. Pedrigi, Leila Towhidi, and Rob Krams

Department of Bioengineering, Royal School of Mines, Imperial College London, London, UK

Corresponding author – Rob Krams, Dept. of Bioengineering, Royal School of Mines, Imperial College London, Exhibition Rd., SW7 2AZ London, UK; phone +44-207-594-1473; fax: +44-207-594-9817; email r.krams@imperial.ac.uk

Abstract

This review provides an overview of the effect of blood flow on endothelial cell (EC) signalling pathways, applying microarray technologies to cultured cells, and *in vivo* studies of normal and atherosclerotic animals. It is found that in cultured ECs, 5–10% of genes are up- or down-regulated in response to fluid flow, whereas only 3–6% of genes are regulated by varying levels of fluid flow. Of all genes, 90%are regulated by the steady part of fluid flow and 10% by pulsatile components. The associated gene profiles show high variability from experiment to experiment depending on experimental conditions, and importantly, the bioinformatical methods used to analyse the data. Despite this high variability, the current data sets can be summarized with the concept of endothelial priming. In this concept, fluid flows confer protection by an up-regulation of anti-atherogenic, anti-thrombotic, and anti-inflammatory gene signatures. Consequently, predilection sites of atherosclerosis, which are associated with low-shear stress, confer low protection for atherosclerosis and are, therefore, more sensitive to high cholesterol levels. Recent studies in intact non-atherosclerotic animals confirmed these *in vitro* studies and suggest that a spatial component might be present. Despite the large variability, a few signalling pathways were consistently present in the majority of studies. These were the MAPK, the nuclear factor-κB, and the endothelial nitric oxide synthase-NO pathways.

Keywords: systems biology, mechanobiology, atherosclerosis, gene deconvolution, shear stress

1. Introduction

This review provides a nonexhaustive overview of studies that were performed to understand the role of mechanobiology in the initiation and progression of atherosclerosis. After providing an introduction to the effect of blood flow on atherosclerosis, arguments will be presented to motivate the use of systems biological techniques to study atherosclerosis. This is followed by an overview of studies performed using microarrays on cultured endothelial cells (ECs) under fluid flow, or ECs obtained from intact tissue. Finally, we will introduce a novel technique used to estimate gene profiles from ECs in atherosclerosis. This review does not include work on microRNAs; on shear stress please refer to other papers for this topic.[1,2]

2. The effect of blood flow on atherosclerosis

Coronary heart disease (CHD) is the global leading cause of death. In the UK, acute coronary syndromes cause ~60% of CHD deaths and lead to ~240 000 hospitalizations each year, incurring direct healthcare costs of ~£1.7 billion annually. The majority of the mortality of CHD is related to the rupture of a thin-cap fibro-atheroma (TCFA). The characteristics of a rupture-prone plaque are that of a large and soft lipid-rich, necrotic core covered by a thin and inflamed fibrous cap. Associated features include a large plaque size, expansive remodelling preventing luminal obstruction (mild stenosis by angiography), neovascularization, plaque haemorrhage, adventitial inflammation, and a 'spotty' pattern of calcifications.[3,4]

Importantly, atherosclerotic plaques do not distribute evenly over the arterial system but are located at predilection sites (side branches, curved segments, and bifurcations) that are caused by changes to the velocity field, most notably velocity vectors of low magnitude and of altered direction are associated with atherosclerosis.[5–7] Although these studies defined a role of biomechanical factors in the 'initiation' of atherosclerosis, later work indicates an important role for blood flow in the 'progression' of atherosclerosis[8–11] and restenosis after interventions.[12,13] Further studies highlighted the possible role of blood flow in determining plaque composition, by recognizing the large cellular heterogeneity in the longitudinal direction[14–17] (Fig. 1). Recently, human pathological and imaging modalities (optical coherence tomography, virtual histology) confirmed these observations by showing that large lipid cores and TCFA predominantly appear upstream of the culprit lesions.[15,18–23]

Figure 1. 3-D reconstruction of atherosclerotic mouse carotid showing large 3-D heterogeneity in macrophage distribution. Red is high and dark is low. Note that blood flow is directed from the left to the right of the page.

To further understand the role of shear stress in plaque formation, we *induced* two different pro-atherogenic shear stress fields (low, nonoscillatory and low, oscillatory shear stress patterns) in a straight vessel of hypercholesteraemic, ApoE$^{-/-}$ mice, and demonstrated that specific shear stress patterns *induced* specific plaque compositions in a high-cholesterol environment. Surprisingly, the low, non-oscillatory shear stress pattern induced TCFAs, whereas the low and oscillatory shear stress induced stable plaques.[24–28] Further studies, revealed that low-shear stress-induced TCFA formation was associated with the presence of adhesion factors,[28] chemokines,[26] and activating factors for macrophages,[25] indicating that the reduction of blood velocity enhanced uptake and activation of inflammatory cells in the plaques. These observations have recently been confirmed in porcine coronary arteries and in human carotid and coronary vessels, indicating that blood flow and its derivative shear stress (which scales linearly to velocity) are fundamental to determining plaque composition.[15,29,30]

In summary, fluid flow plays an important role in the initiation and progression of atherosclerosis. The close association with plaque progression may be explained by the fact that certain velocity patterns determine plaque composition. The important role of blood flow in atherogenesis deserves further attention, and the remainder of the paper describes the underlying mechanism with respect to the mechanosensitive pathways in ECs. Other reviews have been published, describing other relevant mechanisms underlying flow-related predilection sites.[31,32]

3. Mechanosensitive signalling pathways in ECs

ECs are known to have 10–15 (cytoskeleton, Cav3.2T-type channels, stretch-dependent Na^+-channels, stretch-dependent K^+-channels, PECAM1, the glycocalyx, G-receptors, caveolae, cilia, and membrane fluidity) mechanosensors, and several studies have identified that 10–20 signalling pathways are modified by mechanical stimulation. These mechanosensitive pathways regulate 10 acknowledged transcription factors, which lead to the expression of 1000–2000 genes.[33–37] The sheer number of mechanosensitive pathways, their interactions, and their unknown dynamics in ECs covering a plaque offers a challenging problem to identify individual signalling cascades.

Classical physiological studies have investigated relatively few components per study; for example, a standard experimental protocol in cardiovascular research examines single gene functions through the use of transgenic mice. Although interesting and informative, it is now becoming increasingly apparent that such studies are not sufficient to explain complex processes such as plaque initiation and atherogenesis. Even a relatively simple phenomenon, like the formation of a fatty streak, involves multiple cell types (e.g., T-cells, macrophages, and the endothelium) and changes in the gene profile of each cell type, probably involving hundreds to thousands of genes. Probing single proteins will establish their functions, but it will not provide a full understanding of their interactions. For this, a multi-scale analysis in which the activities of all relevant proteins are monitored over time and then integrated into a quantitative mathematical model is required to provide a deeper understanding of these cellular dynamics.

A new branch of biology, defined as systems biology, aims to identify the components of complex systems and to model their dynamic interactions and behaviour.[38–45] This approach matured after the Human Genome Project provided a list of all human genes, which professionalized new technologies such as RNA sequencing, expression microarrays, and tandem mass spectrometric analyses of proteins and metabolites. Given the complexity of the cardiovascular system, and of cardiovascular diseases, systems-based approaches are likely to play an increasingly important role in elucidating the higher-order interactions underlying traits such as atherosclerosis, cardiac hypertrophy, heart failure, and arrhythmias.

This paper provides an overview of studies that apply high-throughput techniques and bioinformatics to evaluate the effect of blood flow on the initiation and progression of atherosclerosis. This topic was chosen because it is emerging as a new perspective in understanding the pathogenesis of atherosclerosis and it is becoming increasingly important, for new diagnostics and therapeutic interventions.

4. Microarray studies in ECs

In the last decade, the microarray technology has been used by many investigators to explore genomic changes of ECs in response to mechanical perturbations and after over-expression of genes involved in the regulation of the mechanosensitive signalling cascade (Table 1). *In vitro* experiments were carried out with cells exposed to different shear stress regimes,[37,46–63] co-cultured with vascular smooth muscle cells (VSMCs),[64] virally transduced to over-express mechanosensitive genes[51,65,66] and/or exposed to inflammatory cytokines[52,59] (Table 1). In addition to cell culture experiments, some groups have also studied changes of the endothelial transcriptome *in vivo*.[37,58,61,63,67] These investigations were mainly conducted in pigs, as these animals have similar haemodynamic scales (e.g., Reynold's numbers) as humans. A common strategy to this approach is to firstly assess the fluid flow and associated shear patterns, in different geometrical parts of the main arteries, applying a combination of imaging and computational fluid dynamics (CFD). Through CFD, flow patterns can be modelled and shear stress maps calculated and related to image-derived plaque components.

Table 1. An overview of studies using microarrays to determine mechanosensitive signalling pathways in ECs

Cell type	Shear conditions (dyne/cm²)	Duration (h)	Type of platform	Dgene (%)	Dgene	Ref.	Year	Coverage (%)
In vitro cell culture								
Huvec	0, 25	24	cDNA-1	0.22	52	McCormick et al.[58]	2001	56
Huvec	0, 10	24	cDNA-1	2, 0.8	205/86	Garcia-Cardena et al.[90]	2002	56
HAEC	1, 13	24	Atlas	20	69	Brooks et al.[91]	2002	5
Huvec	0, 25	24	GDA	5.7	230	Dekker et al[57]	2002	20
Huvec	0, 13	24	Agilent	5/5.5	1052/1147	Yee et al.[85]	2008	100
Huvec	0, 12	24	Affy	16/17	3427/4010	Mun et al.[86]	2009	100
Huvec	1, 15	24	Agilent	1	194	Conway et al.[70]	2010	100
Huvec	15, 75	24	Illumina	2	303	White et al.[72]	2011	100
BAEC	0, 15, 75	24	Affy	3.5, 7	303/603	Dolan et al.[73]	2012	43
In vivo ECs in non-atherosclerotic animals								
PAEC	DF vs. UF	NA	cDNA-2	10	2000	Passerini et al.[76], Zhang et al.[80]	2004	65
PAEC	CEC vs. IEC	NA	Operon	0.5	52	Zhang et al.[80]	2008	52
PAEC	Multiple sites	NA	Operon	1.1	133	Civelek et al.[78]	2009	52
PAEC	10, 15, 32	NA	Operon	7	730	LaMack et al.[61]	2010	52
PAEC		NA	Operon	11	1300	Civelek et al.[79]	2011	52
MAEC	LF vs. UF	48	Illumina	2	583	Ni et al.[75]	2012	100
In vivo ECs in atherosclerosis								
MAEC	OSS vs. LSS	6w/9w	Affy	30	6300	Frueh et al.[84]	2011	100

HUVEC, human umbilical venous endothelial cells; PAEC, pig arterial endothelial cells; MAEC, murine arterial endothelial cells; BAEC, bovine arterial endothelial cells; tu, turbulence; cDNA-1, a platform of Research Genetics, containing 11.397 unique genes; Atlas, a dedicated platform, containing 1086 genes. GDA platform has 18 000 constructs. Agilent contains 44.000 60-mers with an unknown coverage. cDNA-2 is a platform of Astra Zeneca and contains 13 824 clones. Operon offers a platform with 10 665 clones. Dgene is a differential gene expression, which is presented as absolute numbers or as the percentage of number of genes spotted on the platform. Susceptible is supposedly low-shear stress region, and atheroprotective a high-shear stress region. Affy, affymetrix; CEC, coronary endothelial cell; OSS, oscillatory shear stress; LSS, low-shear stress; LF, low flow; DF, disturbed flow; UF, undisturbed flow; h, hours; w, weeks. See text for details.

5. Microarray studies in cultured ECs

In 2001, Garcia-Cardena and McCormick et al. were the first groups to address how gene expression in human umbilical venous endothelial cells (HUVECs) changes when exposed to laminar shear stress. The microarrays of McCormick et al. contained only a restricted number of genes, as the entire human genome was not deciphered until 2003. Nevertheless, 52 genes were found to be differentially expressed, of which 32 genes increased upon

exposure to shear stress. The increased gene expression such as cytochrome P450, ASS1, and human pGL transporter are known as markers of atheroprotection. Interestingly, Krueppel like factor 4 (KLF4)4 was shown to be shear responsive at both time points.[58] Genes that play a key role in the initiation of atherosclerosis were consistently down-regulated, such as connective tissue growth factor, which mirrors some of the effects of TGFβ, ET1, and MCP1.[58] Dekker et al. extended these experiments by shearing HUVECs with either steady flow (25 dyne/cm^2) or pulsatile flow (12 ± 7 dyne/cm^2) for 24 h and up to 7 days. Twelve of 18 000 genes were identified with significantly altered expression (more than five-fold), among these, the authors measured that endothelial nitric oxide synthase (eNOS), thrombomodulin, cytochrome P450, diaphorase 4, PECAM1, and regulators of G-protein expression were up-regulated. KLF4 expression could not be detected, but the shear-dependent up-regulation of KLF2 was shown for the first time.[57] In line with the emerging shear susceptibility theory, inflammatory and thrombogenic markers, such as VCAM1,were down-regulated.[57] Chiu et al. expanded the experimental conditions by an additional administration of tumor necrosis factor (TNF)-α after exposure to static, high-level shear stress (HSS, 20 dyne/cm^2) or a low level of shear stress (LSS, 0.5 dyne/cm^2) for 24 h.[54] They concluded that pre-shearing of ECs decreased the TNF-α responsiveness of many pro-inflammatory, pro-coagulant, proliferative, and pro-apoptotic genes, whereas it increased the responsiveness of some anti-oxidant, anti-coagulant, and anti-apoptotic genes. HSS exposure combined with TNF-β infusion led to the expression of anti-oxidants such as eNOS, cytochrome P450, haemeoxygenase 1, thrombomodulin, and KLF4, while inflammatory markers, such as Eselectin, VCAM1, TGF-β, BMP4, and cyclins, were down-regulated.[54,68] Hence, from these early pioneering studies, it may be concluded that shear stress provides an anti-atherogenic gene profile and that these effects are partly regulated through pathways shared with TGF-β.

In 2006, Dekker et al.[51] further investigated the role of KLF2 and used a lentiviral system to constitutively over-express KLF2, without exposure to shear. They could determine KLF2-dependent over-expression of atheroprotective genes, such as eNOS, thrombomodullin, MAPK9, and von Willebrand factor and of pro-atherogenic factors such as nuclear factor-kappaB (NF-kB), BMP4, interleukin8 (IL8), and SERPINE1 were down-regulated.[51] Interestingly, 42 endothelial signature genes, including vascular endothelial growth factor (VEGF)-receptor1/2, Tie-like receptor tyrosine kinase 1/2 (Tie1/2), platelet-derived growth factor, their ligands, and VE-cadherin, were not significantly affected by KLF2.[51] Parmar et al.[69] added complexity to the shear regimes by modelling an atheroprotective and atheroprone shear stress waveform to which HUVECs were exposed for 24 h. They also over-expressed KLF2 with an adenovirus and investigated the response to IL-1β. KLF2 was highly up-regulated in response to shear stress with the atheroprotective waveform. Genes that were down-regulated by KLF2, but not modulated by IL-1β, included Ang-2 and ET1, while nuclear factor of activated T-cells cytoplasmic-dependent (NFATc3), eNOS, and ASS1 were down-regulated. Genes that were up-regulated by IL-1β and antagonized by KLF2 included IL-6, VCAM-1, and tissue factor. Genes that were synergistically up-regulated by both stimuli were PI3K, prostaglandin E synthase (PTGDS) 1 and VEGF. The group further demonstrated with other experiments that KLF2 expression is regulated by the activation of a MEK5/ERK5/MEF2 pathway. Conway et al.[70] compared the transcriptional

profile of HUVECs after 24 h exposure to one of the three shear regimes: reversing shear stress (RVSS; 1 ± 11 dyne/cm^2), HSS (15 dyne/cm^2), and LSS (1 dyne/cm^2).[70] They found that in comparison with static conditions, the RVSS and LSS exhibited a similar pattern. Up-regulated genes were metallothionein 1 and cell cycle genes such as cyclin B3, cytochrome P450, and syndecan 1, whereas KLF2 and natriuretic peptide receptor A (NPR1) were down-regulated. In the high-shear scheme, KLF2 expression was not enhanced, while KLF4, MEF2, and TGFβ were significantly up-regulated.[70]

In 2010, Villarreal et al.[71] addressed for the first time the issue of partially overlapping roles of KLF2 and KLF4 in response to shear stress or an upstream stimulus of constitutively expressed MEK5. They combined over-expression studies of KLF2 and KLF4 and discovered that 59.2% of genes regulated by the activation of MEK5 were similarly controlled by KLF2 or KLF4. These genes were either coordinated in a combinatorial approach or individually independent of MEK5.[71] MEK5/KLF2-dependent up-regulated genes were CD59, 2′, 3′-cyclic nucleotide 3′ phosphodiesterase, and PTGDS, whereas BMP4 was down-regulated. Cathepsin B, sex-determining region Y-box 7, and VEGF were over-expressed in dependence of MEK5/KLF4, whereas INFg-inducible protein 16 was down-regulated. A synergistic up-regulation could be observed for TIE-2, ASS1, nephroblastoma overexpressed (NOV), whereas MCP1 and IL8 were down-regulated.

There might be a subtle difference between genes responding to flow from static culture vs. those that react to varying levels of flow. The first experimental condition could detect non-specifically, up-regulated genes and thereby over-estimate the number of genes that are regulated by flow. Two *in vitro* studies employed multiple flow levels and they have been used to test this idea.[72,73] Unfortunately, despite close similarities in experimental design between both studies, the different microarrays and bioinformatic analyses make these studies difficult to compare. Dolan et al. employed the philosophy that a reduction of the data space [clustering with principal component analysis before applying a gene enrichment analysis (GSEA)] increases the power of the tests, while White et al. just applied a subjective cut-off of 300 differentially regulated genes. As a consequence, Dolan et al. report 132 genes regulated by the different flow levels vs. 303 genes reported by White et al. Dole et al. also showed that more genes are regulated by comparing static to low or static to high flow, suggesting, but certainly not proving, that non-specific genes may be up-regulated by the initiation of flow from a static condition. Among the up-regulated genes in White's study were transcription factors (ATF and KLF), members of the MAPK pathways, vasodilators such as ET1 and COX. Unfortunately, these genes were not up-regulated in Dolan et al., which may be due to different vascular beds (see later), different species, different developmental stages (embryonic vs. adult), and/or the bioinformatics schemes. The resemblance of White's study with previous studies provides strong evidence that the above-mentioned genes are regulated by blood flow.

In summary, the aforementioned studies indicate that ~10% of genes in cultured ECs are induced by a sudden step in flow (from static conditions, Table 1), while fewer genes (3–6%) are regulated by the actual flow level (see studies of White et al. and Dolan et al. in Table 1). These studies differed substantially on a single gene level; however, the nature of the flow-regulated gene signatures in cultured ECs confirms the concept of EC priming by blood flow.[74] In this concept, blood flow confers protection by decreasing pro-atherogenic,

pro-inflammatory, and prothrombotic gene profiles. As priming depends on the actual level of blood flow, one can infer that low–shear stress regions offer less protection at the known predilection sites for atherosclerosis.

6. Microarray studies of ECs obtained from intact vascular tissue

Far fewer *in vivo* studies reported a genome-wide analysis of mechanosensitive pathways.[75–80] One of the first reports, which studied changes in mechanosensitive gene profiles in non-hypercholesteraemic pigs, was conducted by Passerini et al.[76] They compared disturbed flow with undisturbed flow in porcine aortic arches and identified ~2000 genes to be affected by the flow field, applying a platform with low coverage (Table 1). Through a GSEA, they extended the priming concept of flow on ECs.[76] More recently, Zhang et al. investigated the differences between porcine coronary ECs (CECs) gene profiles, which were derived from a more atheroprone vessel, and iliac artery-derived cells (IECs). Comparing the gene expression patterns of CECs vs. IECs, many inflammatory and thrombogenic genes were up-regulated, such as DUSP1, ICAM-1, activated leucocyte cell adhesion molecule, PECAM1, E-selectin, NF-kB, and AP-1. Cytoprotective genes such as members of the homeobox family HOXA10, HOXA9, HOXD3, and superoxide dismutase 2 as well as KLF2 and KLF4were down-regulated. The same group showed that the coronary endothelium has a higher sensitivity to reactive oxygen species (ROS), resulting in more endoplasmic reticulum stress.[79] Similar studies were conducted by Civelek et al., who showed that, next to the effect of flow, a spatial component was present. If they compared the coronary vs. the non-coronary endothelium, ~1300 genes were regulated. This result could be attributed to only the specific properties of the vascular bed. However, as a detailed shear stress field was not determined in these experiments, it cannot be ruled out that differences in flow between coronary and noncoronary sites explain these findings.

A first mouse high-throughput study was performed by the group of Jo et al. They modified the flow pattern in the left coronary arteries and isolated ECs at 12, 24, and 48 h from both the left (intervention) and right coronary (control). They employed a combination of a false discovery rate (FDR) $< 10\%$ and an over-expression > 1.5.[75] Despite these relaxed conditions, they identified only 52 endothelial genes regulated by flow after 12 h, and 583 genes regulated at 48 h, which is a lower number than reported for the porcine studies (Table 1). The reason for this discrepancy is unknown, and may be related to the relatively short time frame of their studies, or the lower reduction of shear stress in their intervention vessel.[75] Interestingly, they identified several previously unknown mechanosensitive genes, such as Lmo4, klk10, and dhh.[75]

In conclusion, in studies performed with the endothelium from intact tissue, flow-dependent priming of ECs was detected. With respect to individual gene signatures, it is too early to conclude that an intact endothelium reacts differentially from their cultured counterparts. Remarkably, the current studies also suggest that in addition to a flow effect, a vascular bed-specific effect is present, leaving CECs more sensitive to ROS-induced endoplasmic reticulum stress than other vascular beds.

7. Microarray analysis of ECs covering atherosclerotic plaques

High-throughput, microarray studies typically utilize measurement techniques based on the RNA of a single cell type, which is removed from its natural environment to avoid confounding influences. Removal of endothelium either occurs early in the process, where the RNA is extracted from cells in culture, or the endothelium is isolated immediately before RNA extraction as in laser capture dissection. Both of these techniques have drawbacks, where the culturing process can cause dedifferentiation of the cells, and the long duration of cell isolation from tissue may cause RNA degradation. This is why we have explored another technique to measure the endothelial genotype called gene deconvolution.

Gene deconvolution is an emerging technique to decipher a cellular genotype in complex tissue.[81–83] The definition of complex tissue is one that contains multiple cell types whose densities potentially change over time—conditions very pertinent for plaque formation. The proposed deconvolution technique assumes that the genes redistribute over the different cell types according to their density and their state of 'activity', and thereby correct for a changing cellular composition of the plaques that enables one to estimate the gene profiles of all cell types present in their 'natural' environment.[80–83] An essential assumption underlying this approach is that nonlinear effects are not present, e.g., that a gene in one cell does not amplify gene expression of other cells. Another assumption is the absence of strong heterogeneity in cellular subsets, which are conditions not met in atherosclerosis. This can be incorporated by adding more (subset) measurements, but since this field is in discussion, we have not included this information in the current approach.

We applied this technique to 24 microarrays obtained from 10 pooled mouse vessels per microarray containing TCFAs and non-TCFAs in a similar vascular segment (for details of the model, see above and Cheng et al.[28]). The proposed technique assumes that the cellular density is known from the vessel segment of interest, and since this was not available, we modified the method by an iterative Bayesian approach to estimate the probability density distribution of the individual cell types from cellular data obtained from other vessels (for details, see Frueh et al.[84]). The deconvolution method has been tested in several prior studies (for details, see Frueh et al.[84]) and it was applied by us for the first time on murine plaque tissue.

We pretreated our microarrays, aiming at minimizing the data space before applying further analysis. We first applied RMA to reduce the number of outliers, diminished gene number by non-annotated genes, low-expressing genes, and non-reacting genes (for details, see Figure 2). As a second step, the iterative Boolean procedure was tested against known cell fractions obtained from a separate series of experiments with a good result, indicating that the method to generate posterior distribution (PDF) of our cellular plaque compositions produced accurate results. Implementing the posterior PDF to the deconvolution method revealed new cell-specific genes that were undetected with the classical SAM method. A first analysis, where time and location were taken as independent variables, revealed that time was not significant. Hence, the time-dependent samples were pooled to increase power of the tests. As a result, we only compare stable vs. nonstable plaques, independent of their development. The number of genes differentially expressed

between vulnerable and stable plaques was 16 645, distributed over VSMCs (1300 genes), ECs (6300), T-cells (4900), and macrophages (5100).

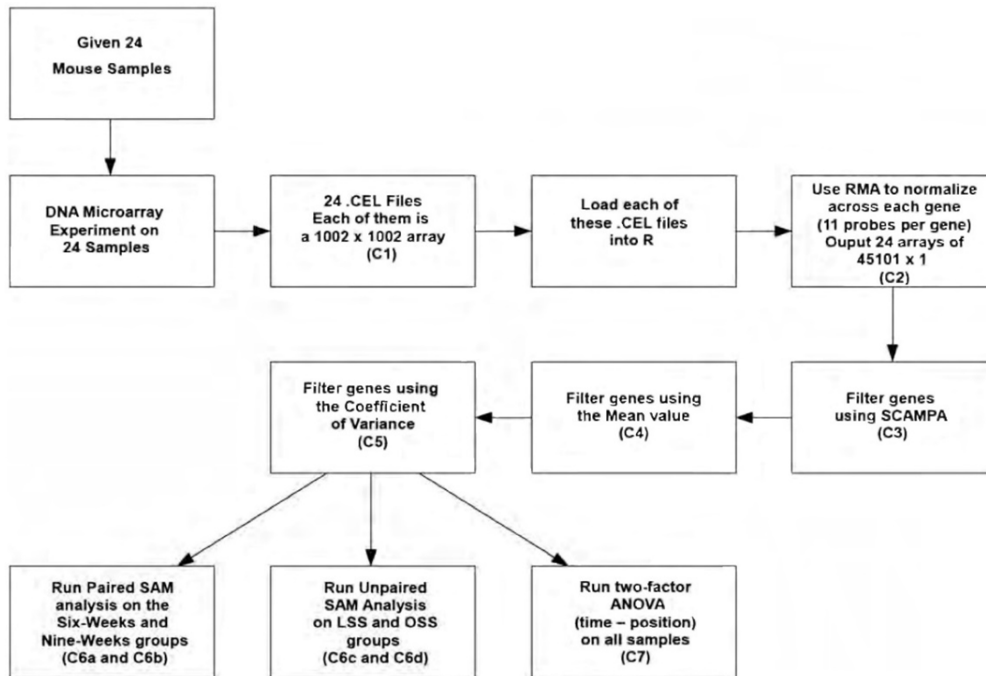

Figure 2. This picture describes the filters used to portray the analysis of the microarrays. After normalizing the microarrays with RMA, we performed a principal component analysis to identify homogeneity of sets of microarrays. Four arrays were found different than the clusters identifying each experimental condition and were deleted. The remaining 20 arrays were filtered using SCAMPA, for low expression using their mean value and for the absence of change during experimental intervention using their variance. The remaining probe set was analysed in several ways using SAM, RANKPROD, and ANOVA.

Pathway analysis was performed by applying GSEA, which identified 49 pathways, 4 of which were activated in both VSMC and ECs, and the remaining 45 pathways were exclusively activated in ECs. In contrast to the studies cited above, which were performed in cultured nonatherosclerotic cells and normocholesteraemic pigs, the preliminary analysis of the endothelial genotype shows a higher content of pro-atherogenic gene profiles with a high activity of the MAPK and PPAR pathways.[84]

8. Bioinformatics analysis as a source of variability

The major aim of this paper was to obtain a robust conclusion on endothelial pathways regulated by flow. However, this appeared a complicated and cumbersome task, as experimental conditions varied among studies, platforms were different, and the bioinformatics was different across studies. While all components may play a role in the variability among the reported studies, a first indication that the bioinformatics appears to play a central role in the high variability among studies, came from a comparison of studies that differed mainly by bioinformatic analysis. Yee et al.[85] and Mun et al.[86] who reported similar experimental protocols, differed only at their choice of platform (Agilent and Affymetrix) and false discovery rates. They reported shear responsive gene regulation to be different by a factor of 3 (1000 and 3000 genes; Table 1). The other two studies,[72,73] which compared two flow levels (15 and 75 dyne/cm^2), showed a two-fold difference in altered gene expression with increasing shear stress (300 vs. 600 genes at 15 vs. 75 dyne/cm^2). However, in addition to a completely different bioinformatic approach (described above), these groups used a different platform with different coverage[72,73] and different cell types.

These first comparisons urged us to study the applied bioinformatics in larger detail. At first instant, it is clear that most studies employed an RMA-like analysis to correct for outliers, followed by an FDR to determine differentially up-regulated genes.[57,67,70,78] However, at closer inspection, one study did not employ an FDR,[65] while others used a combination of ANOVA, degree of expression and FDR,[58] and others combined FDR with fold expression.[84] Furthermore, the level of FDR was different among studies and sometimes varied within a single manuscript. Most studies, subsequently applied GSEA to elucidate signalling pathways. Gene enrichment is appealing but may attribute genes to multiple pathways, providing an over-estimate of pathway involvement.

Recent studies incorporated newer tools to reduce the data space by a stricter preprocessing of data (FARMS[73]), by abandoning nonannotated and nonresponding genes,[84] or by the analysis of subgroups after nonhierarchical clustering.[73] Recently, the argument of having a threshold on the expression level (e.g., FDR) was further relaxed as biological importance is now used as a criterion for significance. Biological importance was deduced from the presence of a network; e.g., whether other genes responded similarly in expression[79] and modules of these networks were further tested for gene enrichment. These studies, in general, report much larger gene regulation by blood flow than the studies using FDR (Table 1).

In summary, it seems that the overall sources of error are wide but could be attributed predominantly to variations in bioinformatic analysis. It seems good to reach a consensus on the approaches used in order to compare the different studies and reach robust conclusions on the presence and regulation of mechanosensitive signalling pathways.

9. Systems biology of mechanosensitive pathways

Despite all of the above considerations, some signalling pathways appeared to be present in most studies. These pathways were the inflammatory pathways (MAPK and NF-κB pathways[61,76]) and anti-oxidative pathways (e.g., the eNOS-NO pathway[58]). Of the

transcription factors, KLF2 and KLF4 were quite often regulated by flow, followed by NRF-2, NF-κB, the ATF-family, p38, and the JNK family.[72] KLF2 and KLF4 have attracted a lot of attention, and they have been reported to be regulated through the MEK5-ERK5 pathway.[69,87–89] New pathways, present in some studies, are the Notch and Wnt pathways. Furthermore, our first study, dedicated to the signature of ECs covering a plaque, indicated a role for the PPAR pathway in the shear-dependent TCFA formation.

In conclusion, several groups have studied mechanosensitive pathways. Over time, these studies became more reliable due to the maturation of the microarray platforms in terms of coverage and repeatability of their responses. During the analysis, it appeared that conformity of the bioinformatics analysis is absent, which introduces a high variability between reported gene signatures. One of the conclusions of this study is that consensus between groups on bioinformatics might increase the usage of previous and new experiments.

Despite these precautions, it seems that ~5–10% of genes are regulated by blood flow, whereas the cultured cell studies indicate that the majority of genes (90%) are regulated by the steady component of blood flow, and only 10% were regulated by pulsatile conditions. The gene signatures could be summarized by a priming concept, where blood flow reduces pro-inflammatory, pro-thrombotic, and pro-atherogenic genes. Of the specific signalling pathways, the MAPK pathways, the NF-κB, and the eNOS-NO pathways were most often mentioned. However, the large variability between the different studies prevents drawing robust conclusions on individual signalling pathways.

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