Sex Differences in Human Aortic Smooth Muscle Cell Phenotypes

Authors:

Sarah Meng, Undergraduate Department of Biomedical Engineering
Rita Anane-Wae, Undergraduate Department of Biomedical Engineering

Advisor:
Dr. Redouane Aherrahrou, Center for Public Health Genomics, Charlottesville, Virginia

Words: 2705
Number of Figures: 6
Number of Tables: 1
Number of Equations: 0
Number of Supplements: 0
Number of References: 23

Signature _____ Date: 05/08/2021
Rita Anane-Wae

Signature _____ Date: 05/08/2021
Sarah Meng

Approved _____ Date: 05/08/2021
Dr. Redouane Aherrahrou, Center for Public Health Genomics

Sex Differences in Human Aortic Smooth Muscle Cell Phenotypes

Rita Anane-Wae[a,1*]. Sarah L. Meng[a,2*]. Redouane Aherrahrou[b]

[a] Department of Biomedical Engineering, University of Virginia
[b] Center for Public Health Genomics, University of Virginia
[1] ra4uz@virginia.edu
[2] sm3bu@virginia.edu
* Authors contributed equally on this work

Abstract

Coronary artery disease is one of the leading causes of death worldwide. This disease is characterized by the buildup of atherosclerotic plaque consisting of including lipids and cellular waste. Smooth Muscle Cells (SMCs) can migrate and proliferate to form a fibrous cap that stabilizes the atherosclerotic plaque in response to plaque buildup. However, in some severe cases, the fibrous cap is unable to prevent plaque rupture which can lead to a thrombotic event causing a stroke or myocardial infarction. Studies have been conducted to identify the genes that are associated with this disease, however, the influence of sex on CAD risk has not been investigated. In this investigation, we explore the differentially expressed genes between males and females as related to atherosclerosis using a unique dataset of human aortic SMC's. We identified a novel *NOTCH4*/*DLL4* pathway that is involved in the differential expression of the genes in males and females. Additionally, we identified the gene *SPARCL1* to be differentially expressed in our dataset. This gene has previously been investigated for its role in atherosclerosis, however, our novel results have shown that this gene, although involved in atherosclerosis, is differentially expressed in males and females, which provides vital information for the mechanism by which this gene functions. Finally, we identified the transcription factors *AR* and *FOXA1*, which have been previously identified to play a role in VSMC calcification. Our investigation, however, provides more insight into how these transcription factors influence Vascular SMC calcification. Overall, this investigation shows that differentially expressed genes between males and females in human aortic SMCs exist.

Keywords: Coronary Artery Disease, Atherosclerosis, RNA-seq, Human Genetics

Introduction

Cardiovascular diseases (CVD) account for 31% of all deaths worldwide. Coronary Artery Disease (CAD) constitutes 43.2% of all CVD.[1] Environmental, lifestyle, and genetic factors contribute to the likelihood of developing CAD. As a complex disease, the genetic propensity to develop CAD depends not only on one genetic variant but on a combination of multiple variants that cohesively increase the risk.

CAD is caused by plaque buildup in the walls of the arteries that supply blood to the heart, which are also known as coronary arteries. The plaque is made up of deposits of cholesterol, fat, and waste that build up in the walls of the artery following damage to the endothelium layer. The plaque buildup causes the inside of the arteries to narrow over time, which can partially or completely block the blood flow. This process is called atherosclerosis. [2] Vascular smooth muscle cells (VSMCs) play a major role at all stages of atherosclerotic plaque development. VSMCs contribute to many different plaque cell phenotypes, including extracellular-matrix-producing cells of the fibrous cap, macrophage-like cells, and foam cells, contributing positively and negatively to the progression of the disease.[3]

Data from the Global Use of Strategies to Open Occluded Coronary Arteries in Acute Coronary Syndromes IIb study has shown that CAD presents differently clinically in males than in females.[4] Typically, females are likely to be diagnosed with CAD 7 to 10 years after males and have a higher expression of cardiovascular risk factors.[5] This sex difference is also seen in other diseases, for example, women with diabetes are at greater risk for cardiovascular complications than their male

counterparts, and low high-density lipoprotein (HDL) cholesterol implicates a higher risk of developing CAD in women than in men.[5] The Genome-Wide Association Studies (GWAS) have identified over 175 loci that are associated with CAD however, their studies have not investigated the contributions of sex-associated genes to CAD.[6]

Thus, the proposed project will investigate differentially expressed genes (DEGs) in males and females using human aortic smooth muscle cell RNAseq datasets. If there are such genes, then therapies could potentially focus on targeting these specific genes or generally prove that men and women would need different treatment plans in treating CAD.

Materials and Methods

This study is investigated with a unique collection of human aortic smooth muscle cells (huASMCs) derived from 118 male and 33 female individuals who either underwent a heart transplant or were victims of motor vehicle accidents.

Cellular and Molecular Characterization of huASMCs

Samples from all 151 donors were cultured in both with-FBS and without-FBS conditions. The with-FBS condition simulated a disease or atherosclerotic environment in which the huASMCs modulated their phenotype to be proliferative. The without-FBS condition simulated a healthy arterial environment in which huASMCs maintained their quiescent phenotype.[7]

We previously investigated the cellular and molecular characterization of the huASMCs. First cellular characterization included investigating 12 CAD-relevant phenotypes expressed by huASMCs including migration (difference in area under the curve, difference in tmax, and difference in slope), proliferation (proliferation response to IL-1B, relative proliferation to IL-1B, proliferation response to PDGF-BB, relative proliferation to PDGF-BB, proliferation response to TGF-1, relative proliferation to TGF-1, and relative proliferation to control), and calcification (calcification in osteogenic media, and calcification in inorganic phosphate media).[7] Next, the cells were molecularly characterized through RNA sequencing (RNAseq), a method which elucidates gene expression information.

The resulting RNAseq dataset is privately owned by the Civelek Laboratory at the University of Virginia and is exclusive to the members of the Civelek Laboratory. This dataset was used to conduct all of the analyses in this project.

Data Separation (Separate Conditions vs Combined Condition)

To conduct downstream analysis, the data was investigated in two separate methods. The with-FBS and without-FBS conditions were first analyzed for sex differences in comparison to each other, for which we have named “Separate Conditions”. Next, the data from the conditions were combined, such that sex differences could be investigated regardless of the treatment condition. This was named as “Combined Condition" for this investigation.

Filtering

Sex Filtering

Before analyzing the RNAseq dataset using the programming language, RStudio, all donors were investigated for their expression of the *XIST* gene (a gene that reveals donor sex). Males in which the gene appeared to be upregulated were removed from the dataset. Females in which the gene appeared to be downregulated were also removed from the dataset.

Filtering by Condition Group

Following this, the data from the separate conditions and combined condition were then filtered to remove outliers. Using RStudio, we identified genes that had 80% or more raw counts that were lower than 5 as outliers and removed them to improve statistical accuracy. We then identified genes located on the X or Y chromosome and removed them using an RStudio package, BIOMART, due to their innate attribution as male or female-specific genes.

Principal Component Analysis

Principal Component Analysis (PCA) through RStudio was utilized in this investigation for multiple purposes, all involving the correlation between the male and female gene expression of the huASMCs. Resultant plots were used to visualize the results of the analysis.

Differential Expression Analysis

DESEQ2

The DESEQ2 software package in the RStudio was one method used to analyze DEGs. With this method, for the separate conditions, a random selection of 30 male donors was compared to all 30 female donors (also known as random partitioning)100 separate times (also known as runs) for each male donor to be analyzed at least once. The DEGs identified in the different runs were then compared

to one another, and the DEGs accrued in at least 50 out of the 100 runs were selected for downstream analysis.

LIMMA

Linear Models for Microarray and RNA-Seq Data (LIMMA) another software package in the R programming language was the second method used to analyze DEGs. The package utilizes linear modeling and empirical Bayes moderation to assess the differential expression and perform gene set testing.[8] Similar to DESEQ2, for the separate conditions we utilized random partitioning with 30 donors for 100 runs for each male donor to be analyzed at least once. The DEGs identified in the different runs were then compared to one another, and the DEGs accrued in at least 50 out of the 100 runs were selected for downstream analysis.

Binding Analysis for Regulation of Transcription Analysis

Binding Analysis for Regulation of Transcription (BART), a tool created by the Civelek Lab and collaborators was used to interpret the DEGs. BART was sourced from the website (www.bartweb.org) and the results were plotted in RStudio.

Results

Quality Control

To ensure that the samples were correctly separated by sex, the gene *XIST* was used as a positive control. Females possess two copies of the X chromosome while males possess one X chromosome and one Y chromosome. *XIST* is a gene that is responsible for X chromosome inactivation in female cells to achieve equal dosage equilibrium with male cells and is only expressed in cells with two or more X chromosomes.[9] The expression of this gene was investigated in the male and female donors.[9] donors were identified to be in the wrong group. As a result, they were filtered out from the analysis to maintain statistical accuracy. In Figure 1, *XIST* is highly upregulated in females and downregulated in males, as expected, and therefore, verified that the samples were then correctly separated for downstream analysis.

Smooth Muscle Cell Phenotypes

12 of the CAD-relevant phenotypes expressed by huASMCs were identified.[7] Of the 12; 7 were specific to the proliferation phenotype, 3 were specific to migration

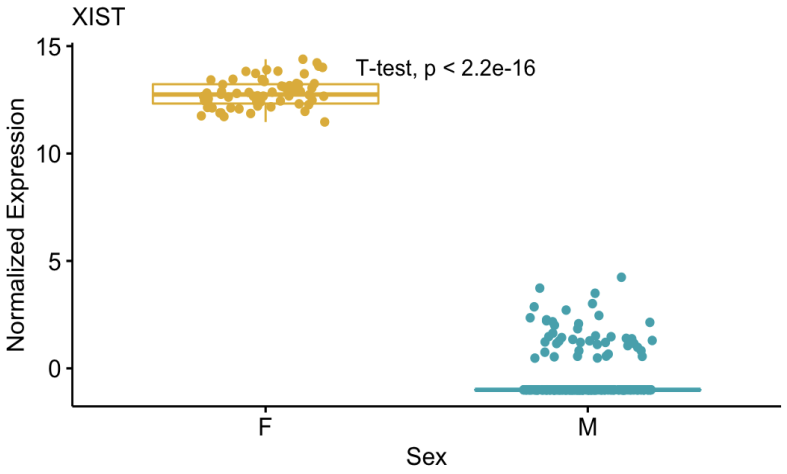

Fig. 1 *XIST* Expression in Males and Females. Results of the quality control that was performed. The gene expression levels of XIST, which is only found in females, was graphed between the female and male samples. The corresponding t-test p-value is shown. Since the p-value is very significant and the gene expression is regulated in the correct direction for both sexes, the samples were correctly separated for sex.

phenotype, and 2 were specific to calcification phenotype.[7] Proliferation refers to the growth or expansion in size and subsequent division of the huASMCs. Migration refers to the movement of the huASMCs from the arterial media to the top of the plaque to contribute to the fibrous cap formation. Finally, calcification refers to the production of calcium phosphate deposits by huASMCs in disease conditions. The twelve phenotypes were analyzed to ascertain the baseline differences in the phenotypic expression of the huASMCs between males and females. The samples were separated by sex for each phenotype and plotted on a boxplot as shown in Figure 2. T-test analysis was then conducted to identify the significance between the huASMCs phenotype in males and females. We determined that the results were significant if the t-test p-value was lower than a cutoff of 0.05. Of the twelve phenotypes investigated, none proved to be significant.

Principal Component Analysis

Preliminary results were obtained by creating PCA plots to investigate possible differences between male and female samples in the with-FBS (disease) condition, without-FBS (healthy) condition, and the combined (healthy and disease) condition using gene expression data as seen in Figure 3. We utilized PC1 and PC2 as they provided the most relevant information. The results from this investigation did not achieve any clear differences between males and females showing that the conditions were negligible.

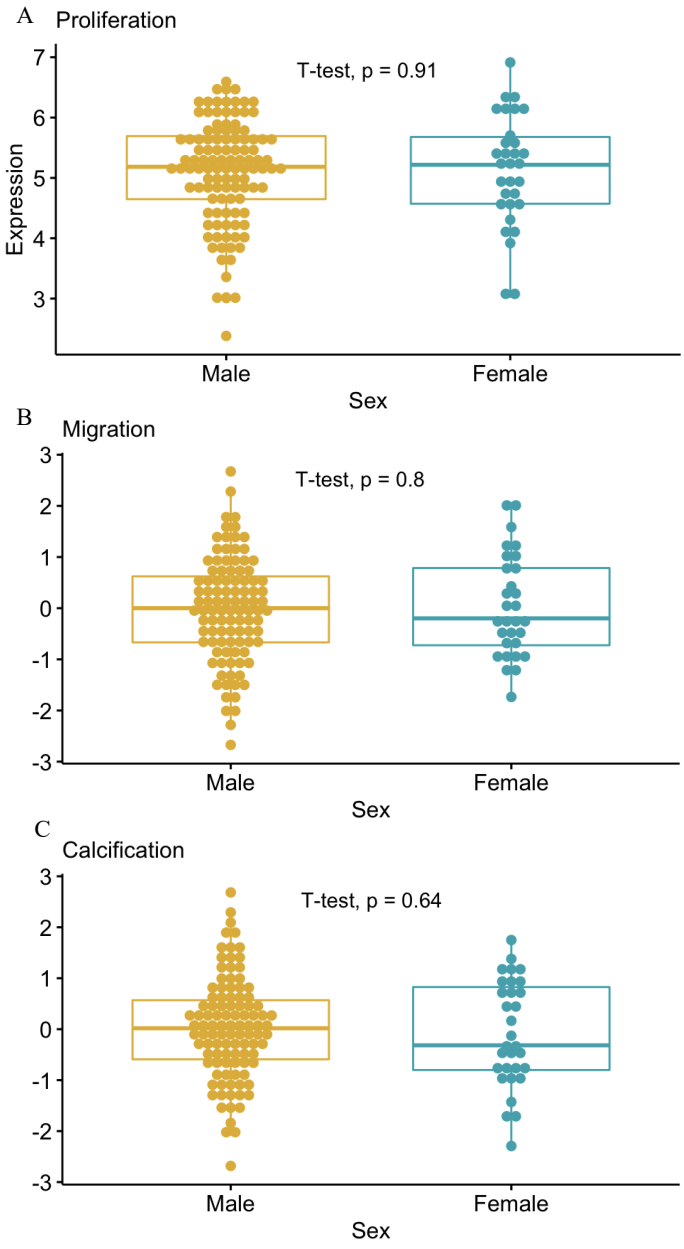

Fig. 2. Representative graphs of the phenotype results. The corresponding t-test p-values are shown. There were no significant results that passed the 0.05 cut-off value. Proliferation (A), Migration (B), Calcification (C)

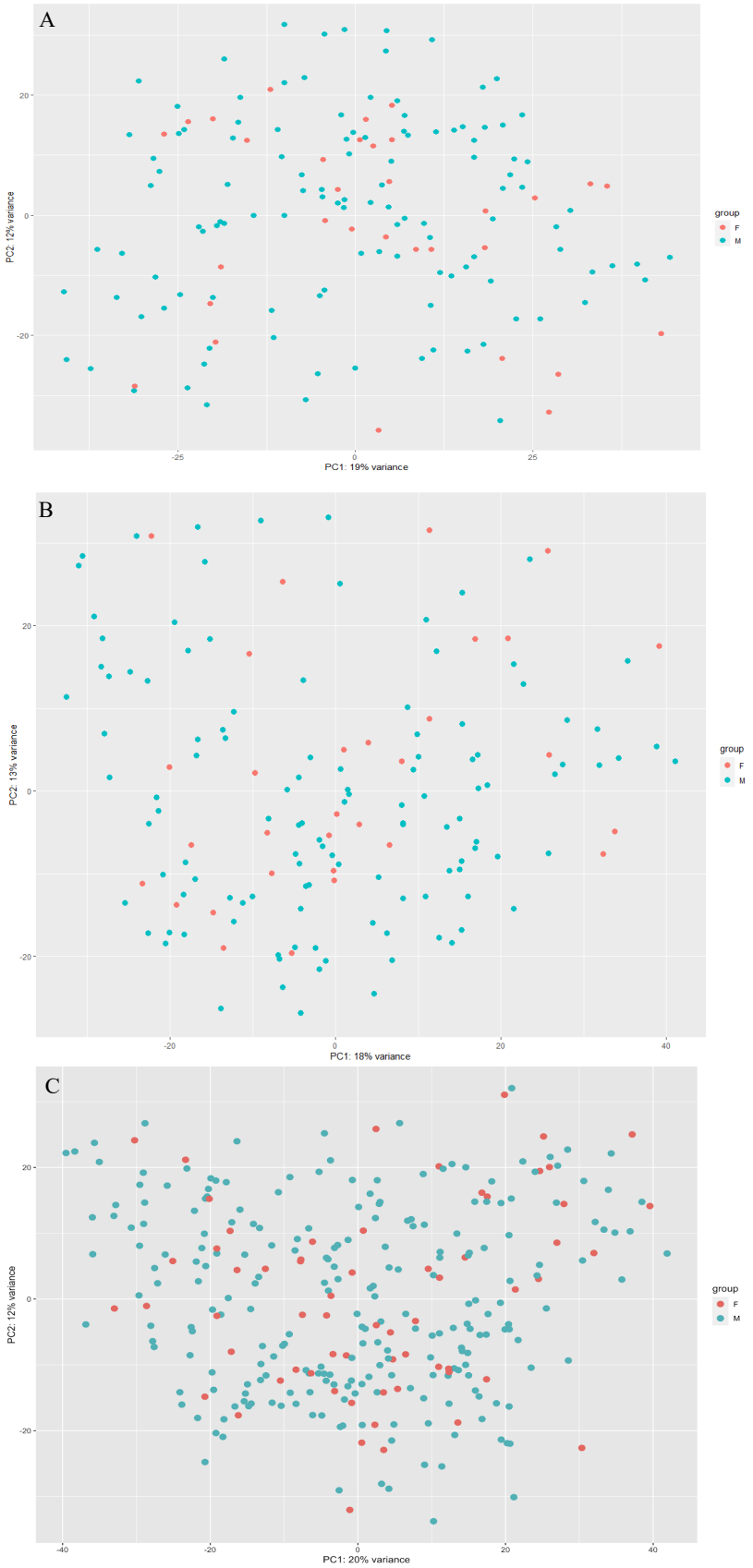

Fig. 3 Principal component analysis (PCA) Plot from male and female, which are shown in blue and red, respectively, human aortic smooth muscle cells in both the with-FBS (A), without-FBS (B) conditions, and joint conditions (C).

DESEQ2 & LIMMA

The DESEQ2 package performs differential gene expression analysis based on a negative binomial distribution.[10] The inputs of this package include count data which is a matrix of genes and their corresponding unnormalized counts for each sample and a metadata file with the phenotype of the cells. [10]Our cohort of 151 human aortic smooth muscle cell donors (142 donors post-filtering) was imbalanced due

to sex. After filtering, 30 female and 112 male donors were retained. As a result of the imbalance, the analysis of the DEGs between sex could not be performed in a typical manner. We, therefore, applied the method of random partitioning to compare the samples in a statistically and biologically significant manner.[11]

Separated Conditions

As a preliminary check, the imbalanced samples were graphed, as shown in Figure 4. There were 53 DEG's in the with-FBS condition and 59 DEG's in the without-FBS condition between males and females when using all 142 donors. Next, we created volcano plots to visualize the DEGs from DESEQ2 as shown in Figure 4. The gray points represent non-significant genes; genes that did not pass either the fold change cut-off or the p-adjusted value cut-off, respectively, are 0.5 and 0.05. The green points represent genes that only passed the fold change cut-off, the blue points represent genes that only pass the p-adjusted cut-off, and the red points represent genes that passed both cut-offs. However, there are more downregulated genes in females, which may be attributed to the smaller number of female samples.

Therefore, we then performed differential gene expression analysis again by applying the previously mentioned random partitioning method using 100 randomly selected cohorts consisting of 30 males and females. The top DEGs are shown in Table 1. We determined genes to be significant if they were consistently differentially expressed in at least 50 percent of the runs. In the with-FBS condition, there was 1 differentially expressed gene (*NOTCH4*) and in the without-FBS condition, there were 4 DEGs (*DLL4*, *SELL*, CHST1, and *NOTCH4*). One gene, *NOTCH4*, was identified to be significantly differentially expressed with both conditions.

Combined Condition

When the two samples (with-FBS and without-FBS) from each donor were combined (to identify DEG regardless of treatment), 4 DEGs were identified. Using this data of 142 donors, we created an additional volcano plot as shown in Figures 5.

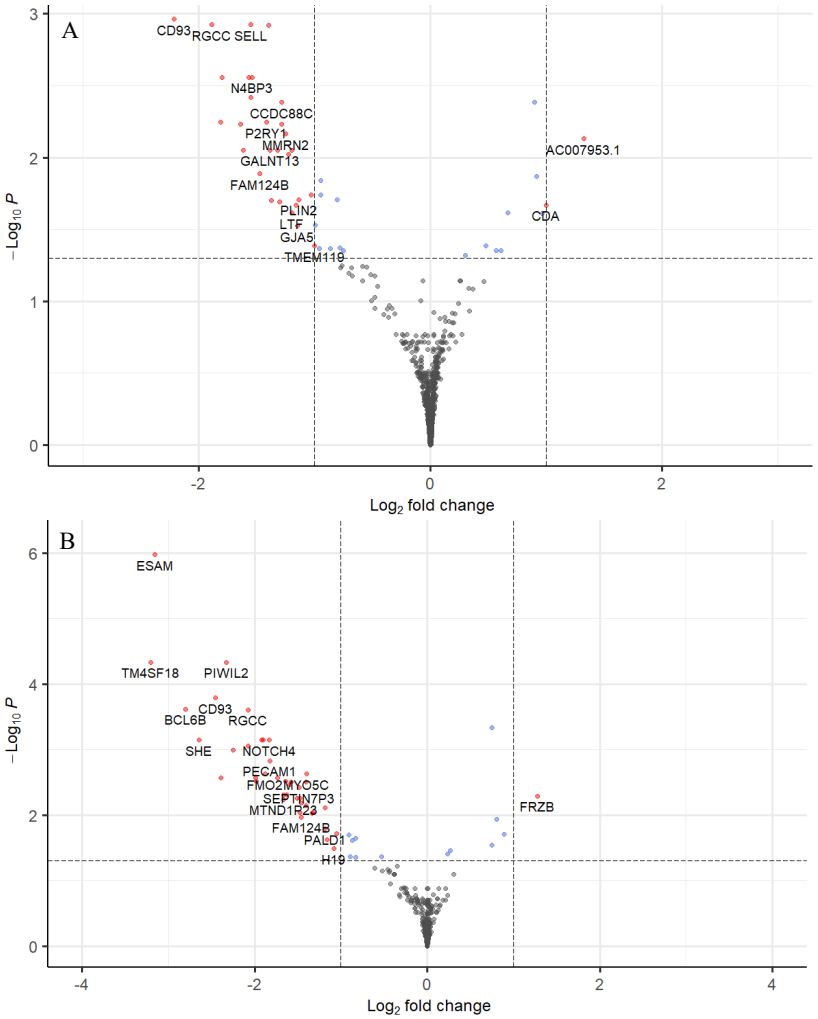

Fig. 4 Volcano Plots of Differentially Expressed Genes in Separate Condition. Plot of the genes in the Without FBS (A) and With FBS (B) conditions. These were made with a p-adjusted cut-off of 0.05 and a fold change cut-off of 0.5. The gray points represent non-significant genes; genes that did not pass either the fold change cut-off or the p-value cut-off. Green points represent those genes that only passed the fold change cut off, blue points represent those genes that only pass the p-adjusted cut-off, and red points are those that passed both cut-offs.

Condition/ Analysis Method	Heathy/ DESEQ2	Disease/ DESEQ2	Combined/ DESEQ2	Combined/ LIMMA
Gene	DLL4	NOTCH4	SPARCL1	GALNT13
Gene	SELL		EIF1AXP1	EIF1AXP1
Gene	CHST1		DKK2	
Gene	NOTCH4			

Tab. 1 Condition, Method, and Differentially Expressed Genes
Differentially expressed genes identified by four different analyses

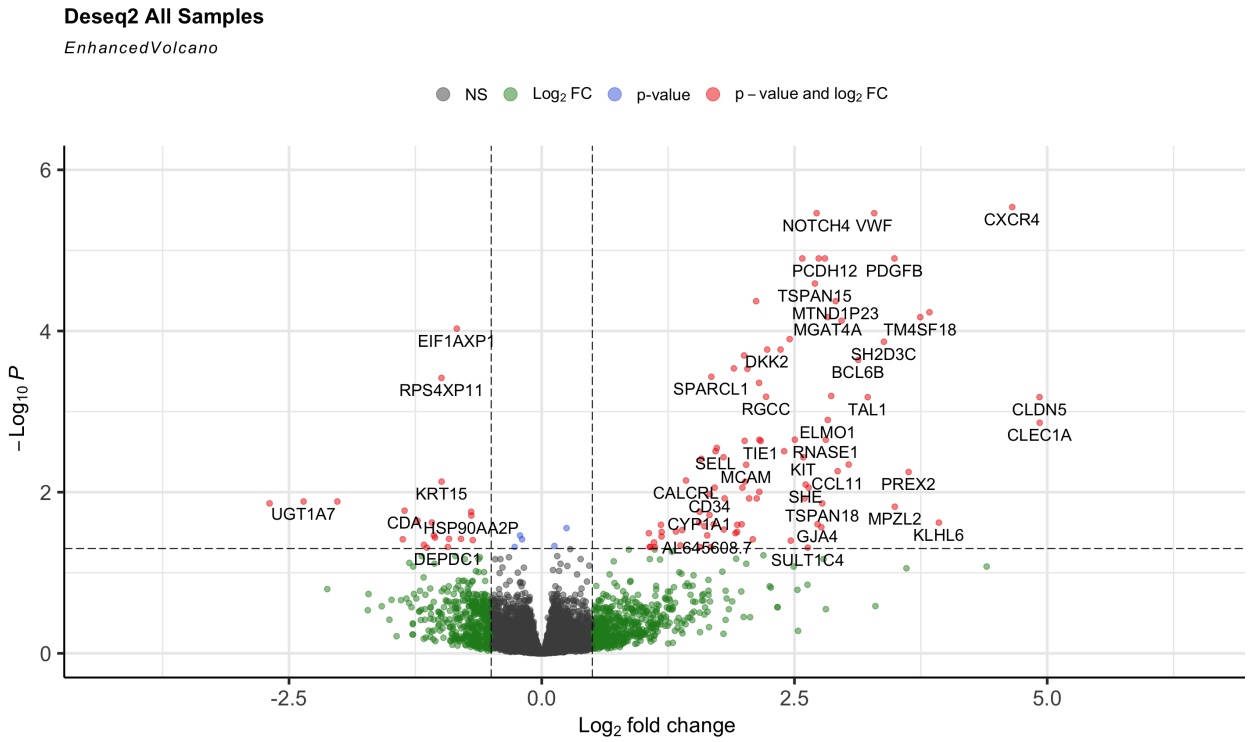

Fig. 5 Volcano plots of the genes in the combined conditions. These were made with a p-adjusted cut-off of 0.05 and a fold change cut-off of 0. 5. The gray points represent non-significant genes; genes that did not pass either the fold change cut-off or the p-value cut-off. Green points represent those genes that only passed the fold change cut off, blue points represent those genes that only pass the p-adjusted cut-off, and red points are those that passed both cut-offs.

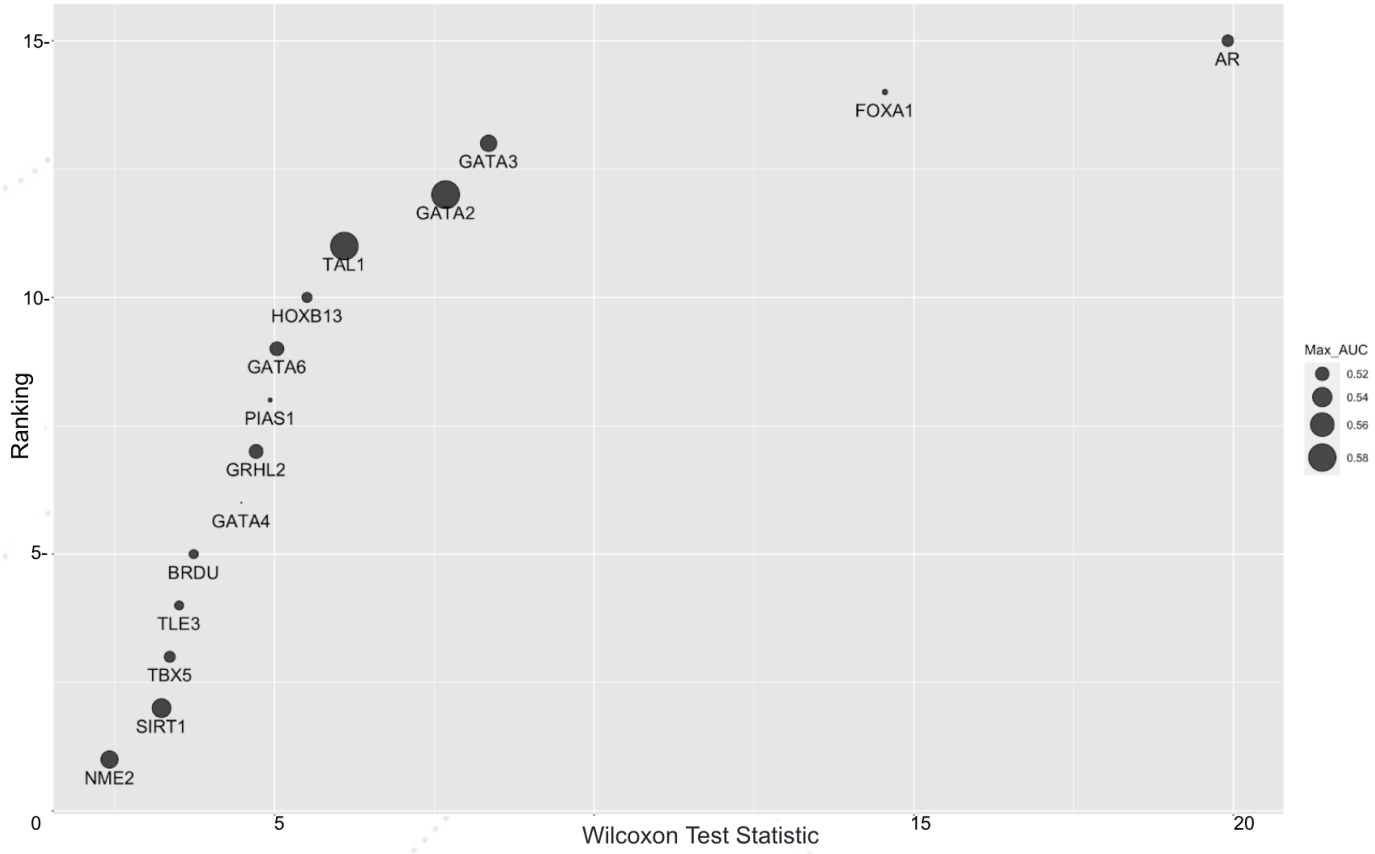

Fig. 6 Results from Binding Analysis for Regulation of Transcription (BART). Transcription factors from differentially expressed genes between males and females. The top 15 significant transcription factors are shown, and they are ranked by Wilcoxon Test Statistic.

We then performed differential gene expression analysis again between males and females' samples using both DESEQ2 and LIMMA. In both methods, we applied the previously mentioned random partitioning method using 100 randomly selected cohorts consisting of 30 males and females. Genes were determined to be significantly differentially expressed in both conditions if, among the 100 runs, they were differentially expressed in at least 50 runs.

Additionally, for the DESEQ2 method, the DEGs had to have passed an adjusted p-value cutoff of 0.05 and a fold change cutoff of 0.5 to be considered significant.

Like DESEQ2, in LIMMA, the DEGs had to have passed an adjusted p-value cutoff of 0.05 to be significant. The significant DEGs with both methods are displayed in Table 1. With DESEQ2, we identified 3 significant DEGs (*EIF1AXP1*, *DKK2*, and *SPARCL1*). With LIMMA, we identified 2 significant DEGs (*EIF1AXP1*, and *GALNT13*). One gene, *EIF1AXP1*, was identified to be significantly differentially expressed with both analysis methods.

BART

To further interpret the identified DEGs, we conducted Binding Analysis for Regulation of Transcription (BART) for the combined condition only. BART identifies transcription factors and chromatin regulators that bind at cis-regulatory regions to regulate gene expression in humans that are also potentially associated with our DEGs.[12] Our fold change cut-off for this method was 1. The functional factor (either a transcription factor or chromatin regulator) name is shown on the y-axis and the Wilcoxon Test Statistic is shown on the x-axis.12 The size of the dot is determined by the max area under the curve (AUC) of the gene.[12] A bubble plot of the top 15 significant transcription factors that were associated with the DEG is shown in Figure 6.

Discussion

Separate Condition

DESEQ2

Overall, there were more differential genes in the healthy condition and most of the downregulated genes are in females. However, the large number of downregulated genes in females could have been a result of the small number of female samples. Therefore, 100 different trials of smaller equal cohorts were run to get rid of the power imbalance between the samples. In the smaller cohorts, there were four genes in the without-FBS condition and one gene in the with-FBS condition that was found to be consistently differentially expressed across the trials. There were fewer genes that were different expressed between males and females in the smaller cohorts. Therefore, it was determined that running the analyses on smaller cohorts was much more representative of the real DEGs than the DEGs identified from using all the donors as the power imbalance was eliminated. That was the reasoning for using this method for subsequent differential gene analyses.

In the With-FBS condition, both *DLL4* and *NOTCH4* were differentially expressed. These genes have been previously shown to play a role in the same signaling network. Both genes were upregulated in males. We hypothesized that the pathway was differentially activated in males and females. *DLL4* plays a large role in vascular development and it is the only *NOTCH* ligand that is expressed predominantly by the vascular endothelium.[13] The *JAG1* gene, which is another player in this signaling network, antagonizes the *DLL4* and *NOTCH* signaling and acts downstream to promote smooth muscle cell differentiation.[13] Finally, *DLL4* inactivation during coronary arterial remodeling results in small arteries, consistent with the fact that *NOTCH4* promotes vascular remodeling.[13] From previous studies linking this pathway to play a role in coronary artery physiology, we hypothesized that this pathway may play a role in the pathophysiology of coronary artery disease that is different between males and females.

Combined Condition

DESEQ and LIMMA

In the combined condition, as expected, there are more differentially expressed genes in the DESEQ2 results as compared to LIMMA, which used a much more stringent/specific approach to identifying DEGs. With both analysis methods, *EIF1AXP1* was shown to be significantly differentially expressed. This gene, which is expressed in aortic tissue, also had the same directionality in aortic tissue from the Genotype-Tissue Expression (GTEx) project (a public resource to study tissue-specific gene expression). Unfortunately, this gene is a pseudogene, and for that reason, we could not utilize it for downstream analysis. [14]

The other significant DEGs include Dickkopf WNT Signaling Pathway Inhibitor 2 (*DKK2*). *DKK2* uses Wingless-related integration site (Wnt) signaling and is involved in embryonic development and breast cancer development.[15]

The transcription factor Polypeptide N-Acetylgalactosaminyltransferase 13(*GALNT13*).[16] Diseases associated with *GALNT13* include Spheno-Orbital Meningioma and Tricuspid Valve Insufficiency.[16] It has however been found to function in the smooth muscle cells of the pancreas.[17] This transcription factor should be further investigated for its expression in smooth muscle cells as related to atherosclerosis.

Finally, SPARC Like 1 (*SPARCL1*) plays a role in the differentiation of pulmonary artery smooth muscle cells through its activation of the BMP signaling pathway.[18] Recent studies have interestingly shown that this transcription factor has heterogeneous expression in cell adhesion and/or cell migration in VSMC's located in the athero-prone aortic arch.[19] Other studies have hypothesized that *SPARCL1* is an extracellular matrix protein that may have anti-adhesive and antiproliferative properties in atherosclerotic vessel walls.[20] This transcription factor should also be further investigated for its involvement in the pathogenesis of atherosclerosis in males and females.

BART

The two most highly ranked transcription factors we identified from the DEGs in the combined condition are both known to be sex-specific. Androgen Receptor (*AR*) is a transcription factor that is involved in testosterone secretion and regulates the development and growth of the prostate.[21] "Targeting androgen receptor in macrophages inhibits phosphate-induced vascular smooth muscle cell calcification by decreasing IL-6 expression" by Pang et al., 2020, explain that they believe they have the first "investigation that has established the correlation between *AR* and [vascular calcification] and identified the contribution of *AR* in the calcification of VSMCs".[22] They state that the exact mechanism behind this process is not yet understood and question the transcription factors' involvement in different sexes, "it has remained unclear whether this discrepancy[(men having more coronary calcification than men)] reflects androgen or *AR* aggravation in men, estrogen or ER (estrogen receptor) protection in women, or a combination of all".[22] As such, our investigation sheds more light on the involvement of *AR* in CAD. *FOXA1* is a transcription that is involved in both the modulation of *AR* in the prostate and (Estrogen Receptor) ER in the mammary gland of females.[23] As our next steps for this investigation, we will explore the role of *AR* in CAD.

Differentially expressed genes between males and females in human aortic smooth muscle cells do exist and therefore, there might be a genetic difference that leads to the risk of coronary artery disease in males and females. However, further investigation of the genetic regulation of gene expression between males and females will need to be performed by methods like expression quantitative trait locus mapping. Further investigation of the significance and regulation of the *NOTCH4* pathway as related to sex differences in CAD needs to be completed as the mechanisms identified in this paper seem promising. Additionally, the gene expression of *SPARCL1* in our dataset presented evidence that it is indeed differentially expressed in males and females. Further investigation of this gene also needs to be conducted. Finally, the transcription factors *AR* and *FOXA1* have evidence that point to a common pathway that affects atherosclerosis differently in males and females. This pathway should also be further explored.

End Matter

Author Contributions and Notes

S.L.M, R.A.W, and R.A designed research, S.L.M. and R.A.W performed research, S.L.M. and R.A.W. wrote software, S.L.M. and R.A.W analyzed data; and S.L.M. and R.A.W. wrote the paper.
The authors declare no conflict of interest.

Acknowledgments

We would like to thank our advisors at the Center for Public Health Genomics, Dr. Redouane Aherrahrou and Dr. Mete Civelek for their endless support and encouragement. We would also like to thank Dillon Lue for his assistance in coding. Finally, we would like to thank the Rivanna staff members for all of their assistance with troubleshooting.

References

1. Benjamin, E. J., Blaha, M. J., Chiuve, S. E., Cushman, M., Das, S. R., Deo, R., de Ferranti, S. D., Floyd, J., Fornage, M., Gillespie, C., Isasi, C. R., Jiménez, M. C., Jordan, L. C., Judd, S. E., Lackland, D., Lichtman, J. H., Lisabeth, L., Liu, S., Longenecker, C. T., Mackey, R. H., Matsushita, K., Mozaffarian, D., Mussolino, M. E., Nasir, K., Neumar, R. W., Palaniappan, L., Pandey, D. K., Thiagarajan, R. R., Reeves, M. J., Ritchey, M., Rodriguez, C. J., Roth, G. A., Rosamond, W. D., Sasson, C., Towfighi, A., Tsao, C. W., Turner, M. B., Virani, S. S., Voeks, J. H., Willey, J. Z., Wilkins, J. T., Wu, J. H., Alger, H. M., Wong, S. S., Muntner, P., American Heart Association Statistics Committee and Stroke Statistics

Subcommittee (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation, 135(10), e146-e603.

2. "Coronary Artery Disease." Centers for Disease Control and Prevention, Centers for Disease Control and Prevention, 9 Dec. 2019, www.cdc.gov/heartdisease/coronary_ad.htm.
3. Basatemur, G.L., Jørgensen, H.F., Clarke, M.C.H. et al. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol 16, 727–744 (2019). https://doi.org/10.1038/s41569-019-0227-9
4. Perdoncin E, Duvernoy C. Treatment of Coronary Artery Disease in Women. *Methodist Debakey Cardiovasc J*. 2017;13(4):201-208. doi:10.14797/mdcj-13-4-201
5. Maas, A H E M, and Y E A Appelman. "Gender differences in coronary heart disease." Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation vol. 18,12 (2010): 598-602. doi:10.1007/s12471-010-0841-y
6. Hughes, M. F., Lenighan, Y. M., Godson, C., & Roche, H. M. (2018). Exploring Coronary Artery Disease GWAs Targets With Functional Links to Immunometabolism. Frontiers in cardiovascular medicine, 5, 148. https://doi.org/10.3389/fcvm.2018.00148
7. Aherrahrou R, Guo L, Nagraj VP, et al. Genetic Regulation of Atherosclerosis-Relevant Phenotypes in Human Vascular Smooth Muscle Cells. Circ Res. 2020;127(12):1552-1565. doi:10.1161/CIRCRESAHA.120.317415
8. Law CW, Alhamdoosh M, Su S et al. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR [version 3; peer review: 3 approved]. F1000Research 2018, 5:1408 (https://doi.org/10.12688/f1000research.9005.3)
9. Ahn, J. & Lee, J. (2008) X chromosome: X inactivation. Nature Education 1(1):24
10. Love MI, Huber W, Anders S (2014). "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." Genome Biology, 15, 550. doi: 10.1186/s13059-014-0550-8.
11. Zhang, Z., Yu, D., Seo, M. et al. Novel Data Transformations for RNA-seq Differential Expression Analysis. Sci Rep 9, 4820 (2019). https://doi.org/10.1038/s41598-019-41315-w
12. Wang Z, Civelek M, Miller CL, Sheffield NC, Guertin MJ, Zang C. BART: a transcription factor prediction tool with query gene sets or epigenomic profiles. Bioinformatics (Oxford, England). 2018;34:2867–2869.
13. Lobov, I., & Mikhailova, N. (2018). The Role of Dll4/Notch Signaling in Normal and Pathological Ocular Angiogenesis: Dll4 Controls Blood Vessel Sprouting and Vessel Remodeling in Normal and Pathological Conditions. Journal of ophthalmology, 2018, 3565292. https://doi.org/10.1155/2018/3565292
14. NCBI. EIF1AXP1 EIF1AX pseudogene 1 [Homo sapiens (human)]. National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/gene/280661. Published May 6, 2021. Accessed April 28, 2021.
15. NCBI. DKK2 dickkopf WNT signaling pathway inhibitor 2 [Homo sapiens (human)] . National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/gene/27123#:~:text=our%20findings%20demonstrate%20that%20DKK2,Wnt%20signaling%20during%20breast%20tumorigenesis. Published March 2, 2021. Accessed April 28, 2021.
16. GeneCards Human Gene Database. GALNT13 Gene (Protein Coding). GeneCards. https://www.genecards.org/cgi-bin/carddisp.pl?gene=GALNT13#:~:text=UniProtKB%2FSwiss%2DProt%20Summary%20for,residue%20on%20the%20protein%20receptor. Accessed April 28, 2021.
17. The Human Protein Atlas. Cell type atlas - GALNT13 . Cell type atlas - GALNT13 - The Human Protein Atlas. https://www.proteinatlas.org/ENSG00000144278-GALNT13/celltype. Accessed April 27, 2021.
18. Wang, Y., Liu, S., Yan, Y., Li, S., & Tong, H. (2019). *SPARCL1* promotes C2C12 cell differentiation via BMP7-mediated BMP/TGF-β cell signaling pathway. *Cell death & disease*, *10*(11), 852. https://doi.org/10.1038/s41419-019-2049-4
19. Dobnikar, L., Taylor, A.L., Chappell, J. et al. Disease-relevant transcriptional signatures identified in individual smooth muscle cells from healthy mouse vessels. Nat Commun 9, 4567 (2018). https://doi.org/10.1038/s41467-018-06891-x
20. Tabibiazar R, Wagner RA, Ashley EA, et al. Signature patterns of gene expression in mouse atherosclerosis and their correlation to human coronary disease.

Physiol Genomics. 2005;22(2):213-226. doi:10.1152/physiolgenomics.00001.2005

21. Davey RA, Grossmann M. Androgen Receptor Structure, Function, and Biology: From Bench to Bedside. Clin Biochem Rev. 2016;37(1):3-15.
22. Pang H, Xiao L, Lu Z, et al. Targeting androgen receptor in macrophages inhibits phosphate-induced vascular smooth muscle cell calcification by decreasing IL-6 expression. Vascul Pharmacol. 2020;130:106681. doi:10.1016/j.vph.2020.106681
23. Yang YA, Yu J. Current perspectives on *FOXA1* regulation of androgen receptor signaling and prostate cancer. Genes Dis. 2015;2(2):144-151. doi:10.1016/j.gendis.2015.01.00