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LINKING QUANTITATIVE RADIOLOGY TO MOLECULAR MECHANISM FOR IMPROVED VASCULAR DISEASE THERAPY SELECTION AND FOLLOW-UP

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Cover illustration: Presented as a lenticular, three views representing the thesis are visualized. The unprocessed CTA image is seen when viewed from the left. Histology-validated tissue characterization is seen when viewed from the middle. Systems biology model simulation-derived therapeutic recommendation is seen when viewed from the right. (*For best results, avoid looking at too extreme an angle*.)

Precision Medicine for Cardiovascular Disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To those who suffer from cardiovascular disease, the largest cause of death and disability worldwide.

POPULAR SCIENCE SUMMARY OF THE THESIS

Cardiovascular disease (CVD) exerts a tremendous toll yet remains underserved in terms of steering the right treatments to the right patients early enough in the disease progression to avoid the adverse sequalae that inevitably follow. In this thesis, we pick up the question of how to remove the drivers of plateaued event rates. We hypothesize that tailored therapeutics on an individual basis, roughly analogous to personalized medicine for cancer but applied to atherosclerosis, could increase patient access to promising therapies early enough in the disease progression to achieve their intended benefits.

We first establish whether routine radiology imaging could be analyzed to provide sufficiently granular information to select among therapies with differing mechanisms of action. One may think that a much higher spatial resolution to visualize the cells and molecules themselves would be needed. Our insight stems from the fact that full granularity may not be required; that rough orders of magnitude and directionality suffice. The number of therapy choices used in the clinic is relatively low, certainly much less than the total number of degrees of freedom understood by a biologist. But we merely wish to "double the therapy choices" from on the order of 2-4 choices to instead, say, 4-8. Even this is challenging, so we proceed step-wise, first to do this at the group level and then to see how far we can go to estimate specific patient data that otherwise require a surgical procedure. We use a form of "multi-scale" modeling, starting with tissue characterization validated by microscopy and then proceeding down to the molecular scale. Multi-scale processing also encompasses a temporal dimension, that is, scaling in time as well, to capture disease progression, stabilization, or regression based on the spatial changes.

We also address an important current consideration of whether pathogenesis is optimally described by tissue characteristics that pertain to molecular drivers or by physical quantities (e.g., stress, strain). We study whether a biological approach such as we take here can also capture physics-based considerations or vice versa. Whereas all modeling types are contributory, differing mathematical formulations may process the core information content differently but may or may not be strictly necessary to draw conclusions. We explore this with a case study.

We then address how such individual-level analysis could drive therapy recommendations. We believe systems biology offers the opportunity to represent disease at a granularity that supports simulations; however, sufficiently comprehensive systems biology models to provide clinically relevant analysis have yet t be reported. Nor have there been means to calibrate such models to individual patients. Until now, only relatively small scope or population level models without clear applicability to individual patients have been reported. We seek to address these limitations. In this way, an integrated clinical decision support system could simulate how a given patient would respond to one or more therapies to support a recommendation.

On this basis, we conclude that techniques such as those developed here could enable the goal of better targeting treatments to individual patients and, through that, improve their outcomes and break through the plateau of adverse event rates in society.

ABSTRACT

Objective: Therapeutic advancements in atherosclerotic cardiovascular disease have improved the prevention of ischemic stroke and myocardial infarction. However, diagnostic methods for atherosclerotic plaque phenotyping to aid individualized therapy are lacking. In this thesis, we aimed to elucidate plaque biology through the analysis of computed-tomography angiography (CTA) with sufficient sensitivity and specificity to capture the differentiated drivers of the disease. We then aimed to use such data to calibrate a systems biology model of atherosclerosis with adequate granularity to be clinically relevant. Such development may be possible with computational modeling, but given, the multifactorial biology of atherosclerosis, modeling must be based on complete biological networks that capture protein-protein interactions estimated to drive disease progression.

Approach and Results: We employed machine intelligence using CTA paired with a molecular assay to determine cohort-level associations and individual patient predictions. Examples of predicted transcripts included ion transporters, cytokine receptors, and a number of microRNAs. Pathway analyses elucidated enrichment of several biological processes relevant to atherosclerosis and plaque pathophysiology. The ability of the models to predict plaque gene expression from CTAs was demonstrated using sequestered patients with transcriptomes of corresponding lesions. We further performed a case study exploring the relationship between biomechanical quantities and plaque morphology, indicating the ability to determine stress and strain from tissue characteristics. Further, we used a uniquely constituted plaque proteomic dataset to create a comprehensive systems biology disease model, which was finally used to simulate responses to different drug categories in individual patients. Individual patient response was simulated for intensive lipid-lowering, anti-inflammatory drugs, anti-diabetic, and combination therapy. Plaque tissue was collected from 18 patients with 6735 proteins at two locations per patient. 113 pathways were identified and included in the systems biology model of endothelial cells, vascular smooth muscle cells, macrophages, lymphocytes, and the integrated intima, altogether spanning 4411 proteins, demonstrating a range of 39-96% plaque instability. Simulations of drug responses varied in patients with initially unstable lesions from high (20%, on combination therapy) to marginal improvement, whereas patients with initially stable plaques showed generally less improvement, but importantly, variation across patients.

Conclusion: The results of this thesis show that atherosclerotic plaque phenotyping by multi-scale image analysis of conventional CTA can elucidate the molecular signatures that reflect atherosclerosis. We further showed that calibrated system biology models may be used to simulate drug response in terms of atherosclerotic plaque instability at the individual level, providing a potential strategy for improved personalized management of patients with cardiovascular disease. These results hold promise for optimized and personalized therapy in the prevention of myocardial infarction and ischemic stroke, which warrants further investigations in larger cohorts.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscripts:

1. Karlöf E, **Buckler AJ**, L. Liljeqvist M, Lengquist M, Kronqvist M, Toonsi MA, Maegdefessel L, Matic L, Hedin U. *Carotid Plaque Phenotyping by Correlating Plaque Morphology from Computed Tomography Angiography with Transcriptional Profiling*, European Journal of Vascular and Endovascular Surgery, Volume 62, Issue 5, 2021, Pages 716-726

2. **Buckler AJ**, Karlöf E, Lengquist M, Gasser TC, Maegdefessel L, Matic L, Hedin U. *Virtual Transcriptomics: Noninvasive Phenotyping of Atherosclerosis by Decoding Plaque Biology From Computed Tomography Imaging*. Arteriosclerosis, Thrombosis, and Vascular Biology, Vol. 41, No. 5, May 2021

3. **Buckler AJ**, Wanrooij MV, Andersson M, Karlöf E, Matic L, Hedin U, Gasser TC. *Patient-Specific Biomechanical Analysis of the Fibrous Cap is Enabled by Histologically Validated Tissue Characterization by CTA: A Case Study*. In press, Journal of the Mechanical Behavior of Biomedical Materials

4. **Buckler AJ**, Marlevi D, Skenteris NT, Matic L, Hedin U. *In Silico Model of Atherosclerosis with Individual Patient Calibration to Enable Precision Medicine for Cardiovascular Disease*. Manuscript

Supporting publications with a significant contribution by the student not explicitly included in this thesis but related to clinical applications during the course of study:

- Berg AR, …, **Buckler AJ**, Mehta NN. *Association of S100A8/A9 with lipid-rich necrotic core and treatment with biologic therapy in patients with psoriasis: results from an observational cohort study*. Journal of Investigative Dermatology, accepted May 2022

- Lal BK, …, **Buckler AJ**. *CT angiographic biomarkers help identify vulnerable carotid artery plaque*, Journal of Vascular Surgery, Volume 75, ISSUE 4, P1311-1322.e3, April 01, 2022

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- Murgia A, …, **Buckler AJ**, … Saba L. *Plaque imaging volume analysis: technique and application*. Cardiovasc Diagn Ther. 2020 Aug;10(4)

- Abdelrahman KM, **Buckler AJ**, Mehta NN, et al. *Coronary Computed Tomography Angiography: From Clinical Uses to Emerging Technologies. JACC State-of-the-Art Review*. Journal of the American College of Cardiology. 2020; 76(10):1226-1243

- **Buckler AJ**, Invited Commentary: *Focus on Quantitative Imaging—Real Progress Is Being Made, but Much Is Left to Do*, Radiographics Vol 39 Num 4, p, 977-980, July-August 2019

- Van Assen M, Varga-Szemes A, Schoepf UJ, …, **Buckler AJ**. *Automated plaque analysis for the prognostication of major adverse cardiac events*. European Journal of Radiology 116 (2019) 76–83.

CONTENTS

LIST OF ABBREVIATIONS

ACS	acute coronary syndrome
AHA	American Heart Association
ANN	artificial neural network
APOB	apolipoprotein B
AUC	area under the receiver characteristic curve
avNNet	averaged neural network
BiKE	Biobank of Karolinska Endarterectomy
BMP	bone morphogenic protein
CABG	coronary artery bypass graft
CACS	coronary artery calcium scoring
CAD	coronary artery disease
CALC	macro- (or dense-) calcification
CAS	carotid artery stenting
CCC	concordance correlation coefficient
CDSS	clinical decision support system
CEA	carotid artery endarterectomy
CEC	cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CFD	computational fluid dynamics
CPM	cellular Potts model
CTA	computed tomography angiography
CVD	cardiovascular disease
ECM	extracellular matrix
ECST	European Carotid Surgery Trial
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
EVINCI	Evaluation of Integrated Cardiac Imaging in Ischemic Heart Disease
FC	fibrous cap
FDA	Food and Drug Administration
FDR	false discovery rate
FEA	finite element analysis
FEM	finite element method
FFR	fractional flow reserve
FRS	Framingham Risk Score
FTMS	Fourier transform mass spectrometry
GO	Gene Ontology
GSEA	gene set enrichment analysis
HDL	high-density lipoprotein
HiRIEF	high-resolution isoelectric focusing
HRP	high-risk plaque
HS-CRP	high-sensitivity C-reactive protein
HU	Hounsfield units
IL	interleukin
IPE	icosapent ethyl

IPH	intra-plaque hemorrhage
IS	ischemic stroke
IVUS	intra-vascular ultrasound
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAP	low attenuation plaque
LCBI	lipid core burden index
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LDL	low-density lipoprotein
LRNC	lipid-rich necrotic core
MACE	major adverse cardiovascular events
MATX	matrix, comprised of normal and fibrotic vessel wall tissues
MESA	Multi-Ethnic Study of Atherosclerosis
MI	myocardial infarction
MMP	matrix metalloproteinase
MQ	model quality
MRI	magnetic resonance imaging
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NIH	National Institutes of Health
NIRS	near-infrared spectroscopy
NO	nitric oxide
OCT	optical coherence tomography
ODE	“ordinary” differential equation
PCI	percutaneous coronary intervention
PCSK9	proprotein convertase subtilisin/kexin type 9
PDE	“partial” differential equation
PDGF	platelet-derived growth factor
PET	positron emission tomography
PROMISE	Prospective Multicenter Imaging Study for Evaluation of Chest Pain
PSM	peptide spectrum match
PVAT	perivascular adipose tissue
QIB	quantitative imaging biomarker
QIBA	Quantitative Imaging Biomarkers Alliance
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSNA	Radiological Society of North America
Rx	prescription
SCOT-HEART	Scottish COmputed Tomography of the HEART Trial
SI	International System of Units
STL	standard triangle language
SVM	support vector machine
TCAR	transcarotid artery revascularization
TCFA	thin cap fibroatheroma
TNF	tumor necrosis factor
Treg	regulatory T-cell
Tx	therapeutics

VSMC	vascular smooth muscle cell
WHO	World Health Organization

1 INTRODUCTION

Therapies for cardiovascular disease (CVD) are increasingly available, and diagnostic capabilities are promising. However, CVD remains the largest cause of death and disability worldwide, mainly by myocardial infarction and ischemic stroke from unstable atherosclerosis [1]. In addition to the human toll, it also exerts an exorbitantly high financial burden on society [2]. Whereas the ability to personalize therapy is increasingly possible in cancer, it has not yet been demonstrated in cardiovascular disease, and consequently, both under- and over-treatment remain common. This results in high numbers of patients needed to treat while simultaneously consuming financial resources and causing patients to go through needlessly invasive procedures for the results obtained.

Risk management of cardiovascular patients currently depends on population-based scoring methods such as the Framingham Risk Score (FRS) or secondary prevention in patients with established disease [3, 4], and the development of diagnostics for more precise patient categorization is warranted. Despite discoveries of new predictive plasma biomarkers [5], routine diagnostic methods for identifying individuals and lesions at high risk for atherothrombosis are still lacking [6]. These diagnostic capabilities remain promising but likely will not satisfy requirements for specificity and, in any case, cannot provide location information. To the extent that methods are proposed to assess vulnerable plaque, there remains the issue that just because a vulnerable plaque may be found, the causes are systemic rather than focal. Focal treatment contributes to overtreatment unless the systemic nature of the disease is also assessed across locations. Markers coupled with therapeutic response simulation are needed to evaluate vulnerable patients individually if we are to make demonstrable improvements in outcomes for given societal cost by personalized medicine (Figure 1).

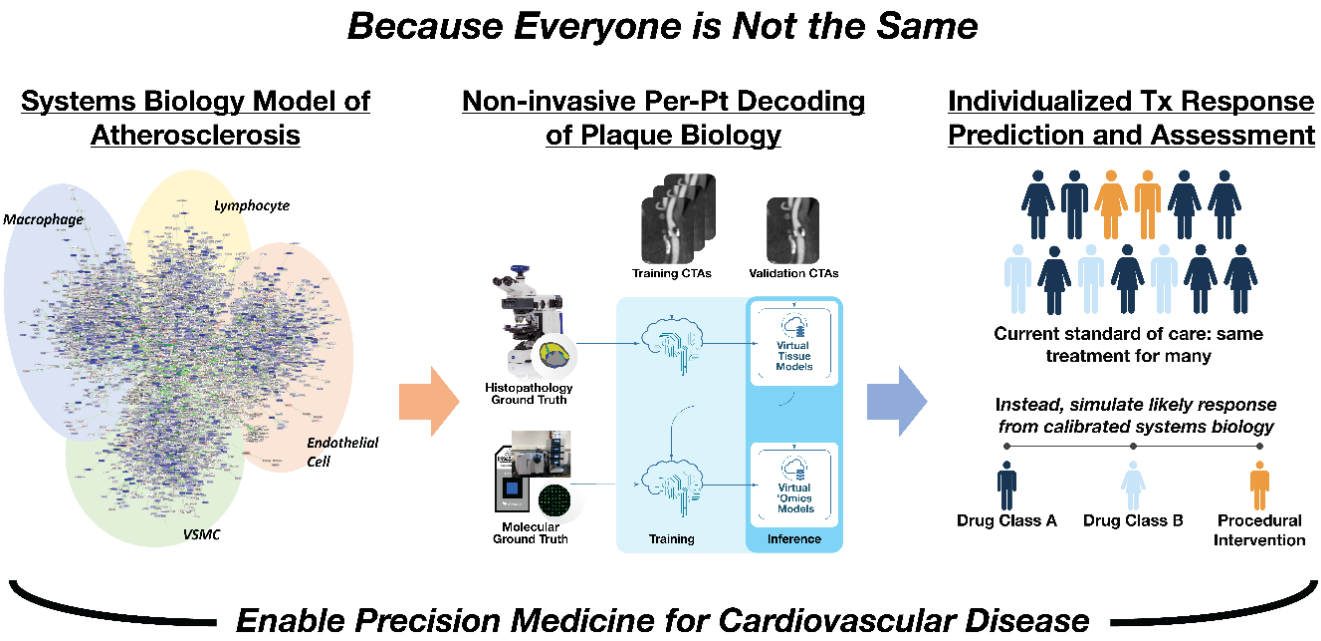


Figure 1: Overall thesis

Each of these needs and opportunities presents challenges to the methods that have so far been developed, with more careful attention needed for rigorously defined markers validated for increasingly powerful indications. Strategies to implement tailored, personalized pharmacotherapy remain limited without practical means to assess biological and molecular disease features non-invasively [7-11]. Specifically, we lack biomarkers to identify at-risk patients and localize unstable atherosclerotic plaques. Such biomarkers not only need to be sensitive, to avoid missing disease but also specific, to aid in the selection of therapy most beneficial to the individual patient. These could be used to develop and prescribe more effective drugs and make more effective use of procedural intervention. Specifically, there is a significant need to help healthcare providers make therapeutic recommendations tailored to specific patients rather than taking a “one size fits all” approach, given the available and future therapies for CVD.

1.1 EPIDEMIOLOGY AND CLINICAL CONSEQUENCES OF CARDIOVASCULAR DISEASE

According to the World Health Organization (WHO), CVD encompassing carotid, coronary, and lower extremity artery disease is the leading cause of death and disability globally [12], mainly by myocardial infarction and ischemic stroke from unstable atherosclerosis [1]. New treatments have been revolutionary over the last 30 years, yet CVD still exerts an exorbitantly high financial cost [2], with a $320 billion annual burden on the U.S. economy alone [13]. This situation is exacerbated by aging and changing ethnic mix [14, 15], as well as affecting an increasing proportion of people globally as economic development continues to narrow the gap between the developed and developing world populations.

In the U.S., the American Heart Association (AHA) projects that over 9% of adults are at more than 20% risk of adverse events within ten years and over 25% more are at significant risk [16], amounting to 23 million high-risk patients and 57 million moderate-risk patients. O these, approximately 30 million U.S. patients are currently on statin therapy in an attempt to avoid new or recurrent adverse events, and the 16.5 million with a current CVD diagnosis are almost all on maintenance medications [17-20].

1.2 RISK FACTORS

Aging: Age is often identified as a risk factor for atherosclerosis, but consensus on the exact mechanisms has not converged. While atherosclerosis takes time to develop and manifest symptoms, not every older person suffers the disease. Differences in genetic predisposition and environmental factors complicate the degree to which age is a causal risk factor vs. merely being the way we experience the development time of the disease. That said, much work has been done to isolate factors that would be strictly age-specific. Factors studied include increasing fibronectin [21] and mutations in hematopoietic stem and progenitor cells [22-26].

Hypertension: Given that atherosclerosis is often triggered by blood pressure, the incidence of atherosclerosis increases with hypertension. Mechanisms that have been studied include increased increase in permeability of the vessel generally and insinuation of lipoproteins into the intima specifically [27-29], as well as enhanced monocyte adherence [30]. Other stretch effects include vascular smooth muscle cell (VSMC) activation, which produces more superoxide [31-33], activating cytokine pathways [34], among other effects.

Sedentary Lifestyle: It is received wisdom that exercise improves cardiovascular health. Specific mechanistic explanations are increasingly documented, for example, as providing stimulus to improve endothelial function as measured by vasodilation [35-37] or by increasing endothelial nitric oxide synthase (eNOS) and other beneficial species [38-41] (directly or indirectly through increases in shear-stress [42]). Interestingly, exercise has also been documented to reduce restenosis caused by catheter injury [43, 44].

Hyperlipidemia: Likewise, there is little doubt that hyperlipidemia is directly involved with atherosclerosis. Apolipoprotein B (APOB) is produced in the liver and is a component of multiple types of low-density lipoproteins (LDL). APOB-containing lipoproteins are

increasingly proatherogenic as their size increases [45]. Conversely, high-density lipoprotein (HDL) is generally considered atheroprotective. Several inter-related mechanisms are involved in atherogenicity, including effects on circulating white blood cells [46], production of cytokines [47], and calcium transients in endothelial cells [48] as specific examples. Hypercholesterolemia, in particular, is hypothesized to decrease the availability of beneficial nitric oxide (NO) [49]; affect relevant signaling pathways [50, 51]; and reduce glycocalyx volume [52]. There is a growing consensus that hypertriglyceridemia vs. hypercholesterolemia differs [53].

Diabetes: The endothelial response and consequent dysfunction in diabetes patients generally, and hyperglycemia specifically, is well known as a comorbidity to atherosclerosis [54, 55], particularly in type II [56]. Endothelial cells with high glucose [57-59] lead to apoptosis and excess reactive oxygen and nitrogen species (ROS and RNS, respectively) which in turn consume beneficial NO, increase proinflammatory fibronectin, trigger premature endothelial senescence, express inflammatory cytokines, and predispose to thrombosis [60].

Autoimmune and Inflammatory Disease: Autoimmune diseases, including lupus [61], rheumatoid arthritis [62, 63], systemic sclerosis [64], and psoriasis [65-67], are also known to be comorbid with CVD. Likely this stems from elevated plasma cytokines [68, 69], which activate endothelial cells or monocytes, initiating atherogenesis, especially in the presence of other aggravating factors. Additionally, regulatory T-cell (Treg) deficiency, immune system dysregulation, and efferocytosis (clearing apoptotic debris) contribute to both autoimmunity and atherosclerosis [60].

Cigarette Smoking: Smoking stresses endothelial cells through multiple mechanisms, apparently through endothelial activation due to oxidative stress causing generation of free radicals, mitochondrial dysfunction, upregulation of heat shock proteins, stimulating effects of nicotine on the vasa vasorum, and cell cycle arrest [70-73].

1.3 CAROTID ARTERY DISEASE

According to the WHO, stroke accounts for 10% of all deaths across the globe, at least 5.5 million deaths annually [12]. Of the approximately 800,000 annual strokes in the U.S., 87% are ischemic. Approximately 15% of all strokes are heralded by a transient ischemic attack (TIA) [74, 75]. Many ischemic stroke events are caused by carotid atherosclerosis [76]. 2.3 million patients in the US are believed to have clinically significant carotid stenosis (>50%), 19% of which are 70% and over [77]. Stroke also results in enormous costs for society, accounting for $36.5 [78] to $74 billion annually [79], estimated to reach $2.2 trillion by 2050 [80, 81].

1.4 CORONARY ARTERY DISEASE

According to the WHO, “coronary heart disease is now the leading cause of death worldwide. It is on the rise and has become a true pandemic that respects no borders” [12]. Of the approximately 1.2 million annual coronary attacks in the U.S., ~66,000 are new, ~305,000 are recurrent, and 160,000 are silent myocardial infarctions (MIs) [74, 75]. Coronary heart disease

caused by atherosclerosis is the most common type of heart disease, killing 365,914 people in 2017 [82]. The relative risk levels for varying degrees of obstruction remain equivocal, with some reports seeming to support the notion that clinically non-obstructive coronary artery disease (CAD) harbors more high-risk plaque than more occlusive plaques, whereas others suggest that the stenotic plaques do have higher event rates [83-89]. In any case, CAD results in over $320 billion in annual healthcare costs and lost productivity, growing by 2030 to $818 billion and lost productivity costs of $275 billion [82].

1.5 ATHEROSCLEROSIS DISEASE MECHANISMS

The underlying pathogenesis often but not always starts with circulating blood lipid species causing dysregulated cholesterol metabolism, which evokes an inflammatory response. However, it may also be caused more directly by systemic inflammatory insult from conditions such as diabetes, psoriasis, or rheumatoid arthritis. Regardless of the initial cause, the response may cascade to structural changes that further accelerate atherogenesis in a positive feedback loop. The morphological and biological features of atherosclerotic plaques have also been corroborated by molecular pathway analyses of the human plaque transcriptome [90, 91].

Atherosclerosis shares a common etiology across arterial beds [92-95]. Carotid atherosclerosis has been shown to predict major adverse cardiovascular events (MACE), even before a diagnosis of CAD [96]. Studies such as the Multi-Ethnic Study of Atherosclerosis (MESA) [97] suggest that this is due to shared triggers and course of pathogenesis. Plaque stability is primarily determined by tissue characteristics rather than age or narrowing, which is similar across arterial beds [98]. The differences that do exist are in the prevalence, rather than the character, of plaque features and do not imply differences in the nature of components when present [95]. Regulation of vascular tone affects plaque evolution across sites [99]. For example, low endothelial shear stress stimulates similar development in both coronary [100] and carotid arteries [101].

While atherogenesis usually takes decades to develop, an interesting and illustrative manifestation is that when veins are used in bypass grafts, they develop atherosclerosis at a relatively rapid pace. Exposing a venous graft to arterial biomechanical loading results in atherogenesis, apparently due to evolutionary differences between arterial and venous tissues [102].

1.5.1 Hemodynamic and Vessel Wall Structural Factors

Atherosclerotic plaque is thought to develop in part from repetitive injury of the intimal surface resulting from oscillatory biomechanical forces of blood pressure. Wall tissues exhibit distributions of stress and strain both the result of, and/or causal for, molecular and cellular changes in the tissues [103]. Hemodynamics has been extensively studied (e.g., [104]), where in conduit vessel blood is most commonly described by a Newtonian viscosity model. Plaque tends to be located at areas of oscillatory or disturbed flow, particularly where there are momentary flow reversals [105, 106]. Such regions demonstrate low shear stress, particularly where vessels bifurcate, that grow based on a combination of factors [107-109]. Blood vessel

response to shear stress is a progenitor of atherogenesis via interacting biological and mechanical factors. Hemodynamics does not cause the disease but can predispose specific sites to the molecular mechanisms that properly constitute the disease [110].

Whereas many analytical approaches proposed for diagnostic applications focus on hemodynamic simulations, they generally neglect the analysis of heterogeneous vascular wall tissues (e.g., [111-113]). We and others believe that disease phenotype and causal mechanism determination are needed to optimize patient care. The biomechanical properties of soft tissues generally, and arterial walls specifically, have been studied to understand stress and strain levels in vascular tissues (e.g., [114-116]). Stress along the circumference generally dominates, exerting forces much larger than the radial stress due to blood pressure. Complex non-linear, anisotropic, and time-dependent biomechanical properties of vessel walls arise from differing tissue characteristics. These characteristics include, among others, concentrations of elastin, collagen, and smooth muscle cells. Hyperelastic constitutive modes may describe vessel wall tissue components under assumptions of homogeneity [117, 118]. Limited efforts are reported to model heterogeneous material properties, generally with idealized rather than actual vessel wall geometry [119, 120]. *Ex vivo* histopathology studies have been attempted to understand the contribution of different tissues to the propensity to rupture using biomechanical analysis [121-125].

1.5.2 Molecular Pathology of Atherosclerosis

We utilize phenotyping systems defined at the microscopy scale to exposit how macroscopic disease presentation ties to the molecular and cellular level mechanisms that cause that presentation.

There have been two major typing systems utilized by cardiac pathologists, Stary and Virmani. Stary's pragmatic definition of plaque regression [126, 127] has been adopted by the AHA's Committee on Vascular Lesions as a classification of histologically defined lesion types to help inform practicing clinicians. Virmani has refined this with scholarship on the relationship of specific presentations to events, including erosion, rupture, thinning of the fibrous cap, and pro-coagulation [89]. This system was updated in [87], which noted that further development would be possible once modalities like those developed in this thesis. These two systems and other terms used are summarized in Table 1.

***Table 1**: Plaque Stability Typing Systems*

	Stary System	**Other terms for the same lesions**	**Virmani System**
Minimal Disease	Initial lesion (type I)	Fatty dot or streak, early lesion	Intimal Xanthoma
	Progression-resistant lesion (type IIb)		Intimal Thickening
	Progression-prone lesion (type IIa)		

Stable Plaque	Intermediate lesion / pre-atheroma (type III)		Pathological Intimal Thickening, Healed
	Calcific lesion (type VII)	Calcified plaque	Calcified nodule, Chronic Total Occlusion
	Fibrotic lesion (type VIII)	Fibrous plaque	Fibro-Calcific Plaque
Unstable Plaque	Atheroma (type IV)	Atheromatous plaque, fibro lipid plaque	Ulcerated IPH TCFA Rupture
	Fibroatheroma (type V)		
	Lesion with surface defect and/or hematoma/ hemorrhage and/or thrombotic deposit (type VI)	Complicated lesion, complicated plaque	

The sections below review molecular mechanisms thought to characterize each of the three major presentations of the disease. It is noted that the presentations are not strictly progressive in that once at one presentation does not mean it is guaranteed to progress to the next; instead, it is possible to remain at the given presentation, skip ahead to a more clinically significant presentation, or regress back to one which is less clinically significant based on a complex interaction between harmful and compensating beneficial mechanisms. This is at once the difficulty, as well as the opportunity, of diagnostics to optimize therapy planning on a per-patient individual level; innovation starts from understanding the factors involved and how they progress mechanistically.

Initiation of Atherosclerosis: Atherogenesis often begins when lipid species, notably LDL, enter the artery wall, which is in turn made possible by activated endothelium cells stemming

from one or more of the risk factors described above. This triggers actions likened to response to injury, causing the proliferation of VSMCs [128] as a form of hyperplasia (Figure 2).

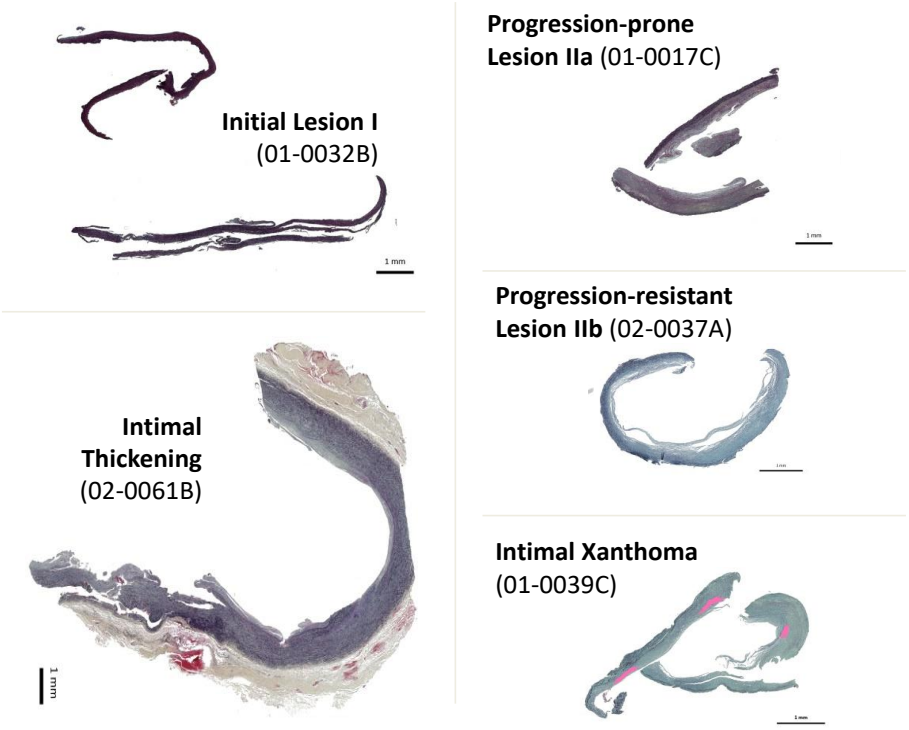


***Figure 2**: Minimal Disease Presentations of Atherosclerosis Initiation. Samples selected from the clinical study Non-invasive Computer-Aided Phenotyping of Vasculopathy (Q-CAMP), NCT02143102 (sponsored by the student). Movat staining. Fragments representing subclinical phenotypes are general pieces at the end of more clinically significant specimens. Neovascularization in Xanthoma is color-coded in purple.*

VSMC proliferation has long been understood as a hallmark of atherogenesis. Research by our group [129, 130] and others has called attention to vascular smooth muscle cell plasticity [131]. The VSMCs accumulate early at predisposed sites to cause intimal thickening prior to foam cell formation, possibly in response to platelet-derived growth factor (PDGF) production by activated endothelial cells [132, 133]. Monocytes migrate from the blood into the tissue, where they become macrophages as an immune response which begins a pro-inflammatory cascade characterized by a concomitant accumulation of these macrophages, which engulf the lipid species called foam cells, particularly in risk factor-induced oxidative stress [134]. The retention further causes the accumulation of proteoglycans, a class of extracellular matrix (ECM) molecules implicated in inflammation [135]. This causes an immune response that begins a pro-inflammatory cascade characterized by a concomitant accumulation of monocytes which engulf the lipid species, particularly in risk factor-induced oxidative stress [134].

In the Stary system, the initial lesion is said to occur at the onset of hyperplasia. Depending on whether it is predisposed by hemodynamic factors, it may progress to either progression-prone or a progression-resistant lesion. In the Virmani system, the hyperplasia prior to the formation of foam cells is referred to as intimal thickening, and then later xanthoma as the macrophage

foam cells become dominant. Figure 2 provides examples of representative subclinical presentations selected from a multi-center clinical study sponsored by the student.

Numerous cytokines amplify and modulate the inflammatory response during the ongoing recruitment of macrophages and other leukocytes to the subendothelial space. Many cytokines promote leukocyte activation, differentiation, or proliferation while actively inhibiting apoptosis, either considered pro-atherosclerotic or anti-atherosclerotic [136], and operating in innate or adaptive immunity pathways or both [137].

Progression of Atherosclerosis: Once initiated, several different pathways interleave in a complex set of interactions. Collagen fibers confer biochemical stability [138], collagen degradation the converse, with the suggested cause being overproduction of matrix-degrading proteases [139]. Reductions in atherogenic lipoproteins resulting from phospholipid and cholesterol efflux improve stability [140]. Regulation of epithelial to mesenchymal transition involves functional transitions of epithelial cells into mobile mesenchymal cells [141]. Cellular switching between epithelial and mesenchymal phenotypes affects tissue structure [142], affecting plaque stability driven in part by altered matrix metalloproteinase (MMP) production [143]. Regulation of Insulin-like Growth Factor transport and uptake is implicated in at least two pathways in cardiovascular tissues [144]. Homotypic cell-cell adhesion between endothelial cells can pathologically allow lipoproteins, leukocytes, and other species to enter the vessel wall [145]. Regulation of membrane protein ectodomain proteolysis is implicated in many diseases, including atherosclerosis [146].

Bone morphogenic protein (BMP) cytokines may induce ectopic bone formation and affect the regulation of development, cell proliferation, differentiation, and apoptosis in various cell types. BMPs promote angiogenesis and osteoblast-like differentiation in VSMC and other cells [147, 148]. Vascular fibrosis involves proliferation of VSMC, accumulation of ECM, and inhibition of matrix degradation.

Multiple risk factors cause oxidative stress, stimulating angiogenesis as a compensatory mechanism to provide the needed oxygen by introducing micro capillaries sometimes described as immature neovessels and observed as “vascular leak.” Distribution of heme within the plaque may be introduced from either the lumen or the adventitia and may develop into intra-plaque hemorrhage (IPH). ECM organization is central to atherogenesis, with synthesis leading to stable plaques and degradation leading to instability [149, 150]. Neutrophil-mediated immunity [151, 152] may promote an influx of inflammatory monocytes early in the process and later may trigger the secretion of pro-inflammatory cytokines. Neutrophils in advanced atherosclerotic lesions may act in thinning the fibrous cap [153]. Apoptotic process depletes VSMCs, resulting in necrosis, through pro-atherogenic inflammatory responses. Efferocytosis helps to withstand atherosclerosis progression [154].

If atherogenic factors dominate over atheroprotective factors, plaques grow in size. Three basic tissue types start to dominate. ECM and/or fibrosis may dominate; VSMCs may become osteogenic, promoting calcification, first as micro calcification and later as macro-calcification

(CALC); or lipid-rich necrotic core (LRNC) and IPH may dominate. Plaques primarily composed of ECM and/or fibrosis or macro-calcification are generally considered stable, and micro-calcification and/or LRNC/IPH are typically considered unstable. Figure 3 provides examples of representative stable presentations.

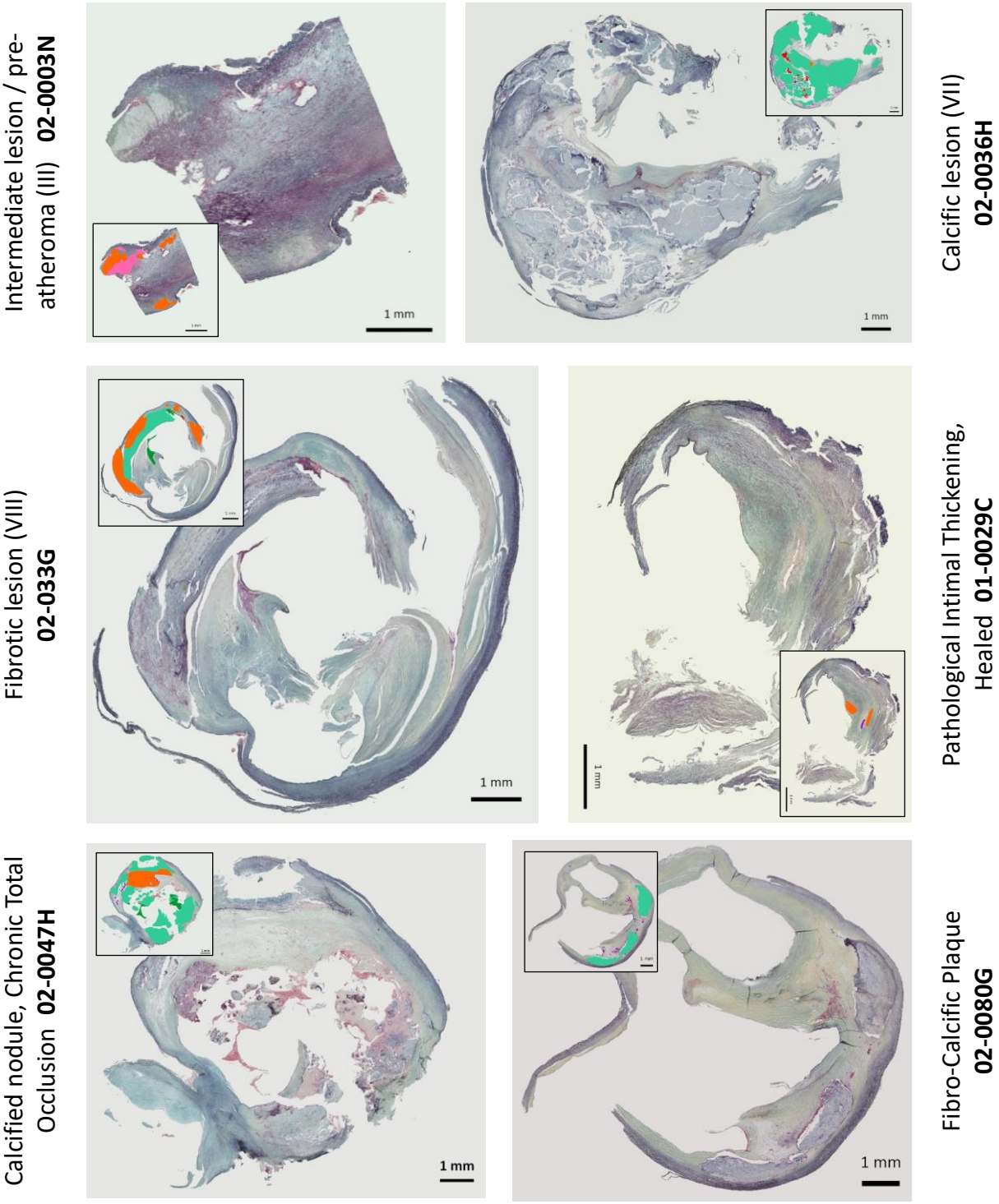


***Figure 3**: Stable Presentations of Atherosclerosis Initiation. Samples selected from the clinical study Non-invasive Computer-Aided Phenotyping of Vasculopathy (Q-CAMP), NCT02143102 (sponsored by the student). Movat staining. Colors: LRNC, yellow; Calcification, aquamarine; Neovascularization, purple; Ulceration, green; IPH, red; Lipid pool, orange.*

Destabilization and Precipitation of Thrombosis: This cellular milieu increasingly causes the development of LRNC [60]. Multiple cell types and debris manifesting varying material densities constitute LRNC, such as lipid deposits, cholesterol crystals, apoptotic cellular debris, macrophages, and calcifications. IPH may comprise intact and ruptured blood cells, macrophages, hemorrhagic debris, fibrin, cholesterol crystals, and calcifications (Figure 4). As the LRNC grows, the previously proliferated VSMCs decrease through apoptosis, degrading the otherwise collagen-rich cap. The cap itself is actually a healing response, similar to the skin encapsulating a splinter.

The unstable atheroma is characterized by several structural- and biological features [155], with greater dependence on the plaque tissues than the luminal narrowing [156-158]. It comes about through increased apoptotic process exacerbated by a hypoxic environment that in turn causes additional angiogenesis. Factors that lead to plaque instability can occur from multiple mechanisms. MMP enzymes and cathepsins are powerful extracellular matrix-degrading enzymes that cause plaque instability. Virtually all migrating cells must secrete some array of digestive enzymes to move through the extracellular matrix. Some MMPs are atheroprotective by promoting VSMC migration and cap formation, but others, such as MMP12, promote inflammation and de-stabilize the plaque [159]. Perivascular adipose tissue (PVAT) has been suspected of promoting increased vascular inflammation from the "outside-in" [160-162].

As the intima thickens, hypoxia induces growth of the vasa vasorum, leaking heme to form unstable plaques. Unstable plaques have more inflammation, accumulation of a large LRNC, IPH, a thin and rupture-prone fibrous cap from matrix degradation and death of VSMCs, and are generally less calcified than more stable, asymptomatic, counterparts [163, 164]).

Outward remodeling and thinning of the media predispose to rupture. Stable atherosclerotic plaques have a thicker fibrous cap [165], which protects the plaque from rupture. Less stable lesions have a thinner cap containing less ECM and VSMCs, often with inflammatory cells, secretion of proteinases, and a growing LRNC. IPH tends to be a marker of unstable rather than stable phenotypes or erosion [102, 166-168]. The observed positive effect of statins indicates that inflammation is also lipoprotein driven [169]. This may lead to rupture or fissure, or, increasingly in the post-statin era, erosion causing mural thrombi distinct from the rupture phenotypes but also dangerous. Figure 4 provides examples of representative unstable presentations.

Ultimately, the release of an embolus (rupture) or an occlusive thrombosis that causes downstream ischemia (or infarction) may result from these advanced, complex plaques. This may progress to acute thrombosis, most commonly via fissure, rupture, or erosion [170]. A mural thrombus localized to the plaque site may be occlusive but remain in a relatively stable presentation referred to as erosion characterized by a "white thrombus" or be considered as a "healed" rupture [171-174].

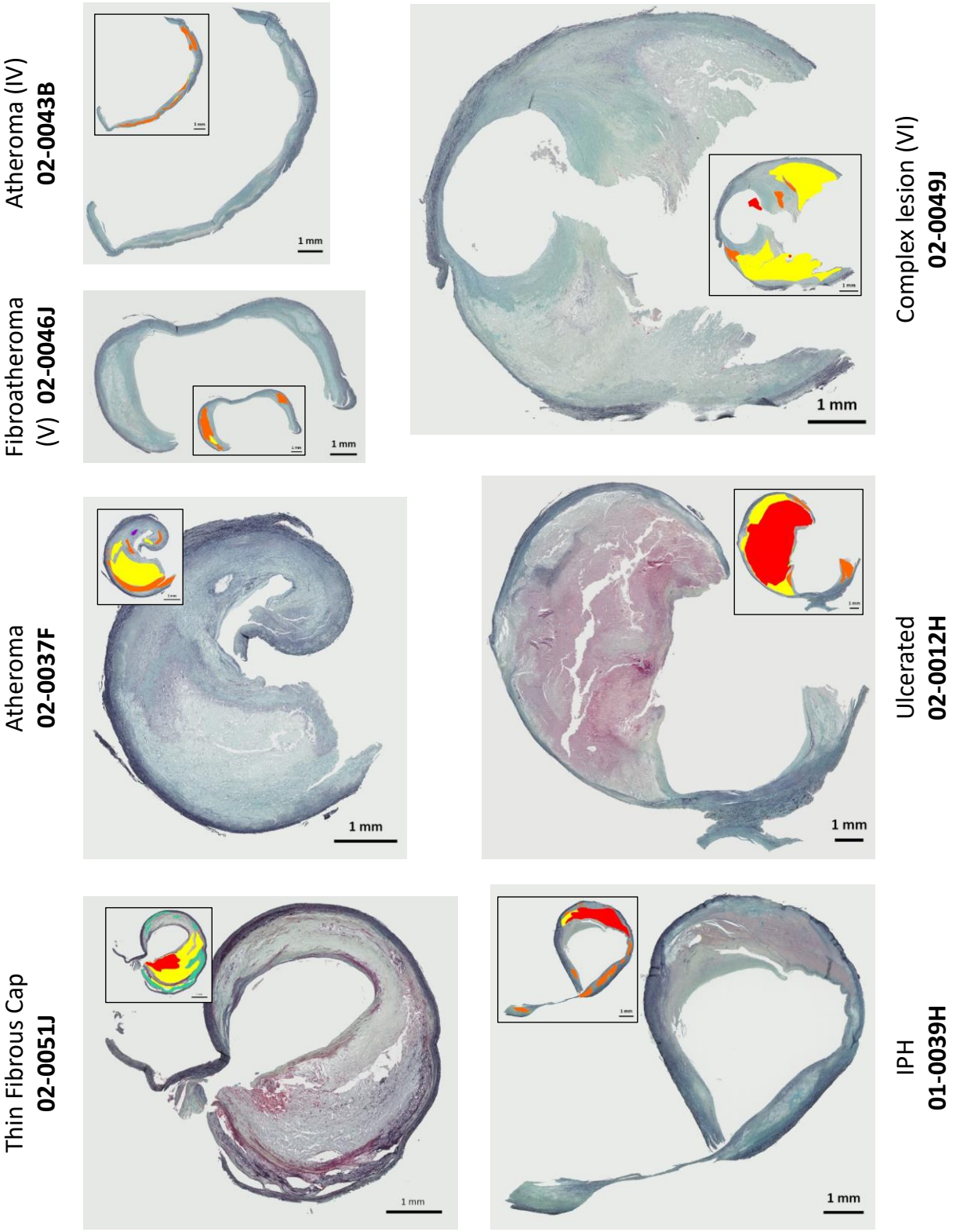


***Figure 4**: Unstable Presentations of Atherosclerosis Initiation. Samples selected from the clinical study Non-invasive Computer-Aided Phenotyping of Vasculopathy (Q-CAMP), NCT02143102 (sponsored by the student). Movat staining. Colors: LRNC, yellow; Calcification, aquamarine; Neovascularization, purple; Ulceration, green; IPH, red; Lipid pool, orange.*

1.6 AVAILABLE TREATMENTS

Statins have slowed but not decreased the growth of the problem. Of the millions of patients on statins, some will develop occlusive disease and acute manifestations, which are often but not always symptomatic. Many questions remain about the best way to treat symptomatic and asymptomatic patients. Procedural intervention versus intensive medical treatment is much debated. The advent of new generation drug classes such as intensive lipid-lowering drugs or monoclonal antibody drugs for inflammation make these questions of urgent importance.

1.6.1 Procedural Interventions in Carotid Disease

Strokes originating with carotid disease are potentially preventable. Treatment alternatives include intensive medical therapies or surgical revascularization procedures (such as carotid artery endarterectomy or stenting) [175-177]. Patients are generally selected for revascularization by the degree of stenosis stemming from a less nuanced understanding of disease mechanisms. Still, evidence continues to grow that this results in unacceptable misclassification [178-180]. In the European Carotid Surgery Trial (ECST), 30% of symptomatic patients who did not undergo surgery with a stenosis of <50% reported stroke or death on follow-up. 6-8 symptomatic patients and 20-30 asymptomatic patients must be treated to prevent one event. There are also questions as to which patients should get carotid artery stenting (CAS) or carotid artery endarterectomy (CEA), particularly with the development of new procedures such as transcarotid artery revascularization (TCAR). Improved diagnostics are required to aid patient selection and procedure planning.

1.6.2 Procedural Interventions in Coronary Disease

Procedural interventions in CAD include coronary artery bypass grafts (CABG), percutaneous coronary intervention (PCI, balloon angioplasty with or without stent placement). Also relevant are valve replacement or repair procedures, including transcatheter aortic valve replacement (TAVR), due to the need for coronary artery assessment in the pre-procedure work-up. Much of the discussion recently has been about the use of these interventions. Despite advances in therapy, trials like COURAGE [181] and ISCHEMIA [182] have not demonstrated reductions in hard outcomes. False positives from function tests are a significant factor in the overuse of invasive coronary angiography and PCI, a major policy issue in the U.S., Great Britain, and China [183-188]. Studies estimate that close to 600,000 of 3.8M annual MPI tests given to U.S. patients with suspected CAD will report false negative findings leading to 13,700 acute coronary events, many of which would be preventable just through the introduction of appropriate drug therapies [189]. Another deficiency of functional testing is temporal: ischemia is a lagging indicator that follows the anatomical changes brought on by disease progression. Patients at high risk for acute coronary syndrome (ACS) would be better served if future culprit lesions could be detected and reduced with intensive drug therapy before the onset of ischemia.

1.6.3 Pharmacotherapy

Most patients on statins are prescribed a relatively low dose, but there are various more intense alternatives depending on how the plaque presents. One approach is to increase the dose; for

example, high-dose atorvastatin is often prescribed for patients with hypercholesterolemia. Another is to better match the drug to the actual condition; for example, there is a growing consensus that hypertriglyceridemia vs. hypercholesterolemia differs [53], with at least one recent drug, icosapent ethyl (IPE, trade name Vascepa), capturing current attention specifically for patients with hypertriglyceridemia specifically. For patients with hypertriglyceridemia, improved outcomes have been reported in trials such as the Reduction of Cardiovascular Events with EPA – Intervention Trial (REDUCE-IT) trial [190-195]. Detailed quantitative studies have yet to be done to determine how IPE affects tissues in the vessel wall because it has not been previously possible to quantitatively assess changes in plaque morphology non-invasively.

Intensive Lipid-lowering Drugs: When biological processes associated with cholesterol metabolism are determined as being dysregulated, various hyperlipidemia control medications may be considered. To the extent that the risk profile is modest, increasing the dosing for broad-based statins, e.g., Atorvastatin, may be indicated. Suppose the plaque markers indicate instability with a necessity for quicker action, but still not at a level that requires instant intervention. In that case, intensive lipid-lowering, e.g., proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors and potentially cholesteryl ester transfer protein (CETP) inhibition, might be indicated, provided the specific mechanism of action markers suggest a response. Hypertriglyceridemia vs. hypercholesterolemia differs, with the former potentially being treated by fatty acid controls, omega 3's, or IPE. Clearing chylomicron remnants (large triglyceride-rich lipoproteins) may be indicated based on the patient's profile.

Emerging drugs, including PCSK9 inhibitors and potentially CETP inhibitors, hold promise; but it has been difficult to quantitatively assess changes in plaque structure and composition with sufficient sensitivity and specificity. The target patient groups are increasingly understood using data from trials such as the Prospective Multicenter Imaging Study for Evaluation of Chest Pain (PROMISE) [196] and the Scottish COmputed Tomography of the HEART Trial (SCOT-HEART) [197]. Based on CTA analysis, patients with non-obstructive but high-risk plaque components can be assigned to the most appropriate high-intensity statin therapy. Still, the real desire is to identify likely responders to even more intensive lipid-lowering strategies that are too expensive to use generally, such as PCSK9 inhibitors. There would be additional diagnostic value in advising whether to add an antiplatelet agent to mitigate the risk of coronary thrombosis and/or to provide longitudinal follow-up to intensify or de-intensify treatment based on the actual response.

Anti-inflammatory Strategies: If inflammatory markers are present but cholesterol metabolism is not evidently dysregulated, the disease may be created by systemic inflammatory insult, for example, by comorbid conditions like rheumatoid arthritis or psoriasis. In such cases, anti-inflammatory drugs, e.g., antibody-based inhibition of IL-1 (canakinumab) colchicine. anti-TNF, anti-IL12/23, or anti-IL17, based on the differentiated signatures from the tissue analysis, or inhibition of the pro-inflammatory cytokines induced on danger signaling. The results may suggest that rather than anti-inflammatory strategies, a pro-resolvin approach may be safer.

Given the concomitant inflammation associated with atherosclerosis, it is natural to consider treating the inflammation. For example, antibody-based inhibition of IL-1 (using canakinumab) has been extensively studied [10, 198, 199]. There have also been ideas for repurposing drugs for other indications which have beneficial effects on plaque, such as colchicine [200]. Patients with other primary diagnoses such as diabetes or rheumatoid arthritis may also suffer vascular disease as a comorbid condition. Treatment by biologic therapy such as anti-TNF, anti-IL12/23, or anti-IL17 has been shown to reduce LRNC [90].

Other highly directed anti-inflammatory therapies may also move to the forefront in the near future [201]. To date, however, there has been a lack of adequately specific biomarkers to identify which patients would benefit vs. which would not, the latter suffering potentially dangerous side effects until or unless a likely response may be established. As a result, these drugs are not yet widely used, despite their apparent promise.

Other Emerging Drug Classes: Triggers of innate immunity and regulation of intracellular signal transduction suggest novel targets for pharmacologic treatment, including inhibiting pro-inflammatory cytokines induced on danger signaling [202]. For example, stimulating immune tolerance with increased Treg activity is being explored [203]. As another example, clearing chylomicron remnants (large triglyceride-rich lipoproteins) [204] is atheroprotective since chylomicron particles are both directly and indirectly implicated in plaque development [205].

Drug candidates in other therapeutic areas, such as immuno-modulators in cancer, can have side effects where atherosclerosis is aggravated due to activation of T-cells in the plaques that can result in plaque rupture. However, accurate methods to track these effects are lacking. There is a widely recognized need for effective markers during drug development for atherosclerosis (and even unrelated diseases) and companion diagnostics post-marketing.

Other therapies such as hypertensives, anti-coagulators, or regulation of intracellular signal transduction may serve the patient best.

1.6.4 Diagnostics for Optimizing Patient Care

While the number of people with atherosclerosis is very high, the fraction of these that will progress to events is small. Most patients are unaware of their disease progression until the onset of symptoms. Risk management of patients largely depends on population-based scoring methods such as FRS [3, 4]. As treatment options for patients with CVD have become available, stratifying patients increasingly needs to be based on per-patient for sufficiently specific determination of individual patient disease category. This is important economically and clinically because recent advances in pharmaceuticals targeting specific mechanisms with increasing efficacy are generally more expensive than earlier generation drugs such as statins and are too costly for use in broad populations.

Yet efforts to develop new therapeutic options to slow or stop the disease are forced to use serum cholesterol, a non-specific marker, or wait for events. Despite discoveries of new predictive plasma biomarkers, such as high-sensitivity C-reactive protein (HS-CRP) or

cholesterol efflux capacity (CEC) [5, 206], and improved plaque imaging [207], routine identification of individuals at high risk for atherothrombosis remains elusive [6]. This makes therapeutic decision-making difficult and increases clinical trials' size and length. We need better answers for selecting patients for surgery and/or tailoring pharmacotherapy. More effective determination of likely response, titrating dose, and longitudinal assessment of actual response, facilitates adaptive decision making to improve patient outcomes and provide healthcare system-wide cost-effectiveness.

The most used imaging guidelines based on stenosis assessment are estimated to have a 30-60% misclassification rate [178, 179, 208-213], resulting in under-treatment (manifesting as high costs for sudden intervention followed by high disability costs) and overtreatment (manifesting as costs of unnecessary procedures and their accompanying risk and complications). Layered on this indeterminate set of biomarkers is the relatively high complication rate of revascularization procedures and incomplete knowledge base of when to intervene surgically vs. when to treat medically. An ability to accurately stratify risk and tailor therapeutics offering even a modest improvement in matching the proper treatment to the patient, holds promise for improving outcomes cost-effectively [214]. Specifically, strategies to implement tailored, personalized pharmacotherapy with a practical, non-invasive assessment of biological and molecular disease features could open the needed innovation in CVD [7].

Imaging Protocols: Significant effort has been and continues to be invested in identifying plaques likely to rupture [98, 215-218] and correlating them with cardiovascular events [219-223], particularly in diagnostic-imaging techniques [215, 218]. Catheter-based imaging has been proposed but, at best, is constrained to invasive procedures given the limited field of view that cannot characterize systemic disease. Moreover, utilization of catheter-based methods inevitably leads to overuse of stenting or other procedures, in themselves good but with increasing evidence of overuse (e.g., [182]) with consequent costs and peri-procedural risks. Effective imaging strategy requires wide-field imaging using relatively inexpensive and available techniques. Catheter-based imaging would never be utilized in patients with sub-clinical or earlier stages of disease progression, even if they could achieve the needed specificity level and image a wide field of view. Our method also builds on earlier efforts that have reached various success levels in non-invasive imaging, e.g. [224-232]. It uses a rationale also tied to the clinical task of informing surgical planning with a more explicit cellular/molecular mechanistic explanation linking morphological presentation of stress-induced ischemia [233, 234], thereby applying to the more acute stages of disease progression.

The modest to low sensitivity and specificity [235-240] of functional testing results in significant over- or under-treatment of CAD. Coronary artery calcium scoring (CACS) has been promoted as a marker for atherosclerosis degree, purportedly being a more specific measure than stenosis. Still, CACS is a lagging indicator that cannot discriminate between the more dangerous micro-calcification and the stabilizing macro-calcification. Positron emission tomography (PET) and magnetic resonance imaging (MRI) are scientifically interesting but do not provide practical capabilities for routine use in the clinic, as does computed tomography

angiography (CTA) [86, 241]. CTA was identified as the most effective in the prospective Evaluation of Integrated Cardiac Imaging in Ischemic Heart Disease (EVINCI) comparison [242].

Another approach to assessing perfusion is to determine the vasculature's ability to transmit oxygen, often expressed as FFR assessed through direct or indirect means [233, 243, 244]. It has been shown in the PLATFORM trial that FFR_{CT} can reduce unnecessary angiograms 83% of the time and reduce the overall cost of care by over $4,000 over one year compared to standard care [245]. Morphological measures of vasodilative capacity may provide the most direct measurement due to LRNC causing dynamic stenosis [243] due to remodeling characteristics and/or the inability for normal signaling needed in response to increased oxygen requirements stimuli [246]. This is exacerbated by inflammatory insult and/or oxidative stress causing endothelial dysfunction [234, 247-249]. PROMISE, SCOT-HEART, and other landmark studies demonstrate the value of CTA in determining the functional significance of stenosis and risk stratification for plaque rupture [83, 196, 197, 233, 250-257].

Formal Classification of Biomarkers: Formal definitions of biomarker type have been promulgated to encourage methodological rigor and discipline in the public discourse on biomarkers and, in particular, to specify testable hypotheses for how they are validated [258]. The following paraphrases this classification system, ordered by the degree of mechanistic specificity needed for the clinical decision:

- A "Diagnostic Biomarker" detects or confirms the presence of a disease or condition, for example, to inform a decision of procedural intervention vs. no treatment or a decision of whether a therapeutic approach may be considered. The latter requires elucidation of what biological processes are dysregulated but may not require whether the condition is causally related to the dysregulation.

For example, LRNC, or a multivariable combination of LRNC, IPH, and wall volume, confirms the presence of vulnerable plaque.

- A "Prognostic Biomarker" is used to stratify individual patient risk of an adverse event in the future, which requires a formalism that captures not only diagnostic differences between individuals but those differences that are of a magnitude to yield different expected outcomes.

For example, CACS does a poor job as a prognostic biomarker. That's because calcification is not all the same; sometimes, it manifests as a stabilizer rather than a destabilizer. But a multivariable model that includes calcification, LRNC, and remodeling ratio demonstrates excellent performance.

- A "Monitoring Biomarker" is a serial assessment of a diagnostic biomarker. This requires that the evaluation be a ratio or interval variable, i.e., not just a "score", because quantitative comparisons are made across points in time. Such a biomarker builds on a prognostic biomarker in terms of the needed mathematical form but with a sensitivity that can not only measure inter-individual differences but, in fact, intra-individual (which is generally more demanding than inter-individual).

For example, LRNC, especially when combined in a multivariable model with cap thickness, identifies not only general but specific responses to both hyperlipidemia and anti-inflammatory drugs.

- A "Pharmacodynamic/Response Biomarker" measures current response after receiving a specific therapeutic. Such biomarkers build on monitoring biomarkers but generally require a model of causation to determine whether the change was due to the particular therapeutic vs. some other reason. Several parameters can be used as a pharmacodynamic /response biomarker depending on the drug being tested.

For example, IPH modulation is tied to patients with hypertriglyceridemia. Quantitative analysis of IPH change, particularly when assessed with a concomitant change in other morphological characteristics, can identify specific response to drug agents that use eicosapentaenoic acid (EPA).

- A "Predictive Biomarker" is used to identify likely future response to a specific therapeutic, generally incorporating a pharmacodynamic model by providing a means to run it out into future cycles from a set of initial conditions.

Whereas a prognostic marker classifies a current phenotype, a predictive biomarker goes beyond that to assert the likely future. Outcome prediction models or drug response simulations fall into this category.

- A "Reasonably Likely Surrogate Endpoint" is an endpoint supported by a strong mechanistic and/or epidemiologic rationale demonstrated to capture a high fraction of treatment effect. Such endpoints build on a pharmacokinetic model, with conclusive evidence that bias and confounding are not limiting generalizability to previously unseen patients of documented characteristics. Surrogate endpoints may be used instead of clinical outcomes in some clinical trials.

An example is total kidney volume for polycystic kidney drugs that have a mechanism of action to decrease the size and number of cysts. This type of biomarker builds on the above to document the specific causal pathway and the degree to which the biomarker is on the causal chain, often represented as a percentage of effect measured.

- "Safety Biomarker" and "Susceptibility/Risk Biomarker" types identify current actual toxicity or likely future adverse reaction. They are counterparts to diagnostic and prognostic markers but where the focus is on off-target effects that occur relative to the intended target but for which assessment is required.

This classification system is most helpful to substantiate a claimed indication for use (or the related concepts of intended use and clinical context of use). Specific statistical and practical considerations must be considered, and data collected and analyzed in particular ways. There may be multiple approaches to implementing a biomarker, whether validated yet or as yet merely putative. Various modeling formalisms may be considered to implement certain types of putative biomarkers. Then, the implemented putative biomarker may be validated according to the type it claims to be. We consider differing model types insofar as their fitness to differing clinical use cases. Full validation would strictly follow the classification system to move from being merely putative to being accepted by regulatory approval pathways.

The development of quantitative imaging biomarkers (QIBs) for research and clinical practice is increasingly important [259-266]. Determining disease subtypes and quantifying severity optimizes patient selection and demonstrates safety and efficacy in smaller and shorter trials. The Radiological Society of North America (RSNA) has organized an activity referred to as the Quantitative Imaging Biomarkers Alliance (QIBA) to convene participants from the National Institutes of Health (NIH), FDA, academia, and industry to develop information, metrology, and other methodological resources for the rigorous development and validation of QIBs [267-276]. These efforts include developing imaging biomarkers for atherosclerosis [277].

1.7 REVIEW OF POSSIBLE APPROACHES TO ACHIEVE THE GOAL

Beginning with the goal of decreasing the event rates of myocardial infarction (MI) and ischemic stroke (IS), several alternative approaches have merit. Whereas the field benefits from pursuing each course, the rationale for the approach taken here may be summarized (Figure 5).

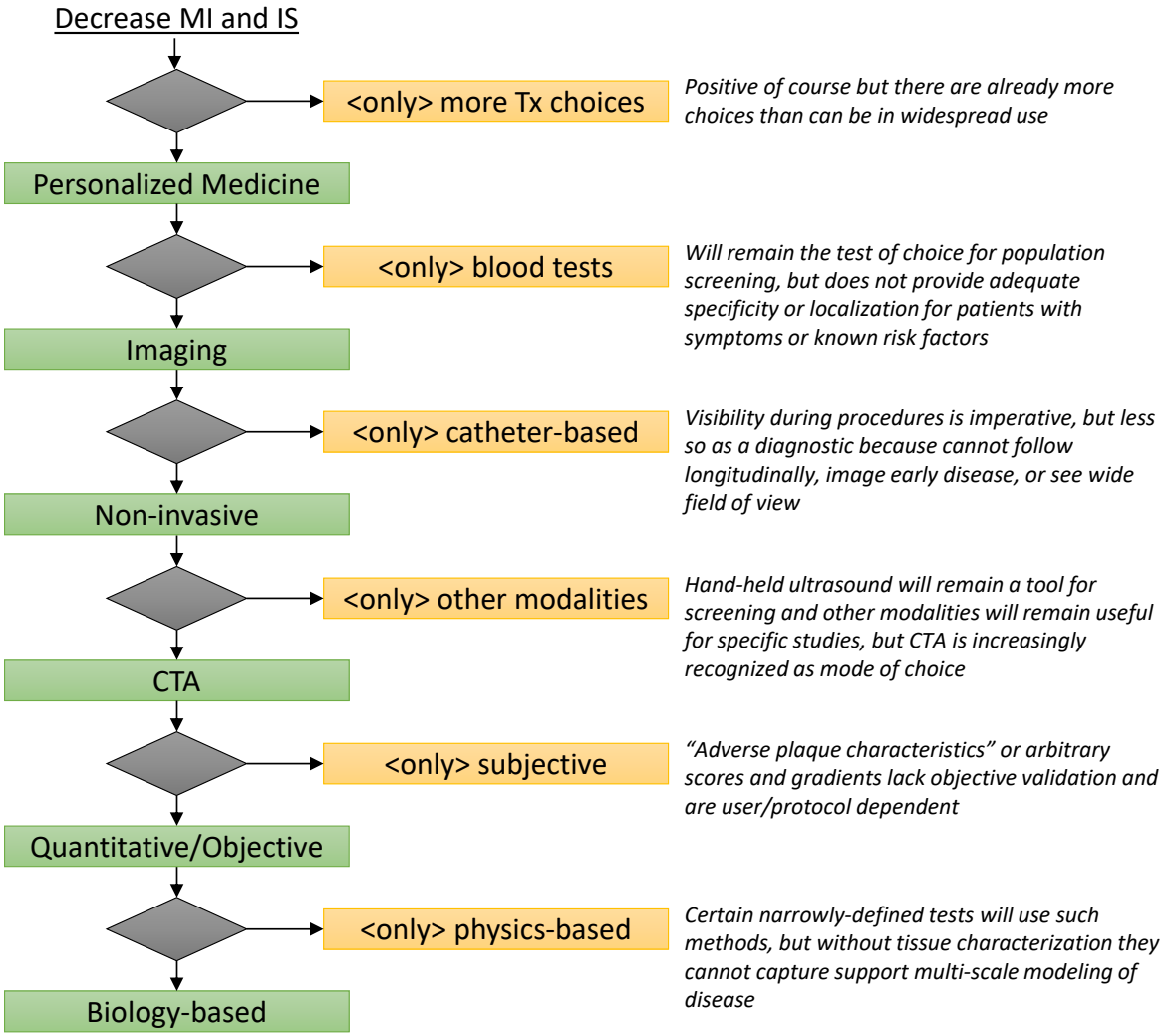


***Figure 5**: Alternatives considered in this thesis to decrease MI and IS rates*

Each is considered below.

1.7.1 Improved Patient-specific Diagnostics

Whereas continued development of additional therapeutic choices is important, the present situation is that not all can be effectively utilized without the ability to target them individually. Therapies for CVD are increasingly available, and current diagnostic capabilities are promising. However, CVD remains the largest cause of death and disability worldwide, mainly by myocardial infarction and ischemic stroke from unstable atherosclerosis [1]. In addition to the human toll, it also exerts an exorbitantly high financial burden [2]. Whereas the ability to personalize therapy is increasingly possible in cancer, it has not yet been demonstrated in cardiovascular disease, and consequently, both under- and over-treatment remain common. This results in high numbers of patients needed to treat while simultaneously consuming financial resources and causing patients to go through needlessly invasive procedures for the results obtained.

Risk management of cardiovascular patients currently depends on population-based scoring methods such as the FRS or secondary prevention in patients with established disease [3, 4]. Developing diagnostics for more precise patient categorization is warranted.

1.7.2 Improved Imaging for Specificity and Localization

Despite discoveries of new predictive plasma biomarkers [5], routine diagnostic methods for identifying individuals and lesions at high risk are still lacking [6]. Likewise, the ability to measure response to drug therapy remains elusive, and both under- and over-treatment remain too common.

1.7.3 Non-invasive Imaging to Rule-in, Not Only to Rule-out

Non-invasive assessment has often been seen as inferior to catheter-based methods, perhaps from the intuition that the closer the sensor is to the tissue, the better the chances of performing analysis. We challenge that notion here, not so much on principle as on practice. First, the incident energy used in catheter-based methods is either intra-vascular ultrasound (IVUS) or light (near-infrared spectroscopy, NIRS, or optical coherence tomography, OCT), rather than the opportunity to use x-rays or radio frequency as may be used in non-invasive scanning (CT and MR respectively). The view that catheter-based methods are a reliable standard may be optimistic [278], particularly when recognition that absolute quantitation in the International System of Units (SI) is generally not possible with catheter-based methods but is possible for non-invasive. For example, such indices as lipid core burden index (LCBI) may be calculated from a 3D modality, and LRNC volume (or even area) may not be calculated by intra-vascular techniques even focally, let alone systemically. Perhaps even more fundamentally, whereas this is possible for x-rays and RF, it remains challenging or even not achievable for either ultrasound or optical imaging.

Various studies have compared catheter-based modalities with histology [279, 280]. Despite the nearly ideal conditions under which these studies are conducted due to being applied on the bench as opposed to *in vivo* and without using multiple pathologist annotators, users, etc., there remain discrepancies between OCT and IVUS [281]. Moreover, the performance data is

generally limited to the lumen rather than tissues within the wall in these studies, or if present, generally only the angular extent rather than volumes or even areas of such tissue.

Consequently, we consider it reasonable to challenge the notion that validation of the non-invasive would require or even favor comparisons with catheter-based methods. Instead, both invasive and non-invasive techniques would ideally be performed against histology; without that, there is no objective standard. Comparison studies can be helpful to leverage the full extent of clinical experience across modalities in the field, but not to identify one as correct versus the other. Such a comparison would require direct comparison with histology in suitably designed study designs that take into account the actual *in vivo* use case rather than only being performed on a workbench from autopsy.

Strategies to implement tailored, personalized pharmacotherapy remain limited without practical means to assess biological and molecular disease features non-invasively [7-11].

1.7.4 Computed Tomography Angiography

The value of CTA is increasingly seen as high [282]. Still, it is currently limited by low specificity and positive predictive value [283] when applied qualitatively or with measures defined from radiology impression rather than which can be objectively determined and which take into account bench science [89]. Objective and quantitative methods are needed to unlock the full potential of CTA. Without such methods, CTA still suffers from overestimated calcification (CALC); subjective and non-specific assessments of low attenuation plaque (LAP) or attenuation gradients which may capture fatty tissues but not necrosis, and in any case, without objective validation; the inability to determine IPH; and observer variability stemming from the use of arbitrary thresholds that depend on both scanner protocol as well as differing across centers. Since necrosis and IPH manifest at overlapping densities, advanced image restoration and tissue characterization techniques enable exquisite assessment.

1.7.5 Quantitative and Objective Imaging Analytics

Atherosclerosis manifests as structural remodeling with a distribution of tissue types. Lipid pools, LRNC, and IPH are all distinct tissue types. Each has differing clinical significance. Atherogenesis manifests as CALC, LRNC, IPH, and intermixed with fibrotic or other tissues rich in extra-cellular matrix (MATX). On CTA, lipid pools are sometimes reported as LAP or attenuation gradients based on their density. But necrosis and IPH manifest higher. IPH and/or proximity of necrotic core to lumen are the most specific markers of plaque stability, motivating the development of methods to delineate them. Sensitivity is relatively more straightforward than specificity in the determination of plaque stability. Reducing event rates requires specific markers such as IPH and LRNC.

Many analysis approaches utilize Hounsfield units (HU) thresholds for separating tissue types on CTA. This analytical method is simple and tends to remain, as cohorts have been analyzed with such software in the past, and converting to a new approach has a cost. The question is, is switching worthwhile? The question comes down to specificity: if the current lower level of

specificity suffices, for example, the clinical need is to rule out, or a judgment on treatment effect may not need rapid assessment, then it may be argued to stay with such methods. However, using simplistic HU thresholds may be holding the field back in multiple ways. In the absence of a histological validation, it remains unclear what the most appropriate values to use for the thresholds. Since differing scan protocols, reconstruction kernels, and subjectivity of the clinical user may affect these numbers, which is exacerbated by the fact that the most important tissue types like IPH to distinguish are not only overlapped in material density but are not always even visually conspicuous.

Moreover, the widely valued measure of cap thickness requires a clear delineation of LRNC, which in turn requires an objective basis to determine the spatial extent, not just the presence, of this composite tissue type. LRNC itself is comprised of a relatively broad and overlapping range of material densities. Even a tissue type considered visually conspicuous like calcification is subject to errors; calcium blooming artifact obscures accurate quantitation and even encroaches on other structures, including the lumen. In such cases, HU thresholding may not provide the needed ability. Objective and quantitative morphology methods are required to unlock CTA's full potential.

Advanced image restoration and tissue characterization techniques enable exquisite assessment. ElucidVivo is the only plaque morphology assessment with histopathologic correlates cleared by the Food and Drug Administration (FDA). Deterministic algorithms preserve mechanistic rationale, and hyperparameters were optimized and validated using best practices. The validation plan and experimental design assessed algorithm performance, accounting for multiple sources of variability. The software produces fully 3D tissue regions at an effective sampling of 3x smaller than the reconstructed voxel size, and 2D cross-sections are sliced from these regions to be in a form comparable to 2D microscopy.

1.7.6 Biology-based as Best Means to Support Multi-scale Analysis

Biological tissue motion, stress, strain, and flow may be modeled in terms of biomechanical or "physics-based" models that draw from analyses often done in inorganic systems, parametrized to approximate the characteristics of the tissues at a given point in time. Finite element analysis (FEA) and computational fluid dynamics (CFD) model the mechanical forces and motions in equations, whether to compute derived terms like strain or shear stress statically or under a time-varying set of boundary conditions.

Generally, the analyses start with a 3D representation of the surface, for example, formatted using methods such as [284] to produce a triangular surface mesh from a 3D volumetric object, such as lumen, wall, or tissue region contours. Morphological processing may simplify surfaces that compromise actual geometry for computational convergence and/or speed benefits. Tissue constituents exhibit differing material properties, which may be approximated by research [124]. Generally, biological soft tissues are slightly compressible nonlinear hyperelastic material properties following the Yeoh model [125]. Fluid properties are chosen to resemble the mechanical properties of blood. In the case of vascular studies, shear rates are generally

high enough to neglect non-Newtonian viscosity phenomena, but non-Newtonian fluid properties may be modeled. Inlet velocities may be approximated or based on Doppler data based on the study's objectives. Pulsatile flow profiles based on heart rate may be applied as a time-dependent inlet velocity field. Time-dependent simulation is done by a segregated solver where the CFD simulation is run with a wall model each time step. The resultant forces at the vessel wall are then applied to the wall, and the displacement field is calculated.

Several specialized models have been defined in terms of specific mechanical interactions as observed in biology, such as the Cellular Potts model (CPM) [285, 286]. Each of these models expresses some form of forces that act in lattices, such as proliferative processes of cells, chemotaxis, or other phenomena of interacting particles, species, or structures.

Whereas one could break down systems biology models painstakingly into discrete physical interactions, one wouldn't use finite element analysis or computational fluid dynamics. These formalisms fundamentally use assumptions of various kinds not met in the more dynamic type of interactions biology manifests. Biological tissue has dynamic rather than static properties, which mutate based on a highly complicated chain of cooperating biological processes with thousands of degrees of freedom generally exceed the granularity at which physicists and engineers approach the problem relative to biologists. Physics-based models may be used in conjunction with systems biology models; for example, given a set of forces computed by a physics-based model, one can use systems biology to model the resulting cell signaling from which dynamic responses occur. Alternatively, given the result of biological evolution, one could use biomechanical analysis to assess the specific set of forces or flow that results at that time. As a result, physics-based models play a role but are not in themselves generally complete or utilized to understand biological systems in their entirety.

Alternative combinatory modeling, including biomechanical and biological descriptors (described as "agent-based modeling"), has also been explored [287, 288]. However, to capture sufficiently granular information, including the prediction of disease-critical biological responses to different drugs, it is necessary to include biological processes represented by pathway networks of molecular interactions essential for disease progression [289-291].

2 RESEARCH AIMS

This Ph.D. thesis expands the link between quantitative radiology and molecular biology, hypothesizing that this novel platform may provide predictive mathematical models for clinical use. To have a clinical impact, it aimed to develop methods using routine clinical imaging to improve decision-support in personalized treatments by being more specific. Improvement in patient care could be achieved with an accurate elucidation of plaque phenotype to realize precision medicine in CVD. A proposed intervention can best address the specific underlying biology. The ability to select the best treatment for those who need it and avoid treatment for those who do not is of increasing focus for achieving quality outcomes with efficient use of health care resources.

Consequently, the thesis aimed to achieve highly specific patient phenotype classification and use it to calibrate patient-specific simulations of drug effects. More specifically, the objectives of this program were to review possible approaches to achieve the goal, devise a strategy based on the review, and conduct necessary projects to establish the feasibility of the selected approach:

I. Confirm that quantitative associations may be made between macroscopic morphology assessed with non-invasive imaging
II. Devise a method to identify molecular-scale levels from non-invasive imaging on an individual patient basis
III. Perform a case study to establish whether biomechanical stress and strain distributions may be determined from tissue characterization
IV. Develop a systems biology model and approach to simulate patient-specific drug response

Significance: Accomplishing the aims of this program results in increased specificity for non-invasive diagnostics and improved detection of unstable atherosclerosis and individuals at risk; more profound use and understanding of plaque biology through linkage to quantitative radiology; and a novel technology platform for drug development and patient management in CVD.

3 MATERIALS AND METHODS

Computational modeling techniques express relationships spanning multiple time- and spatial scales (Figure 6).

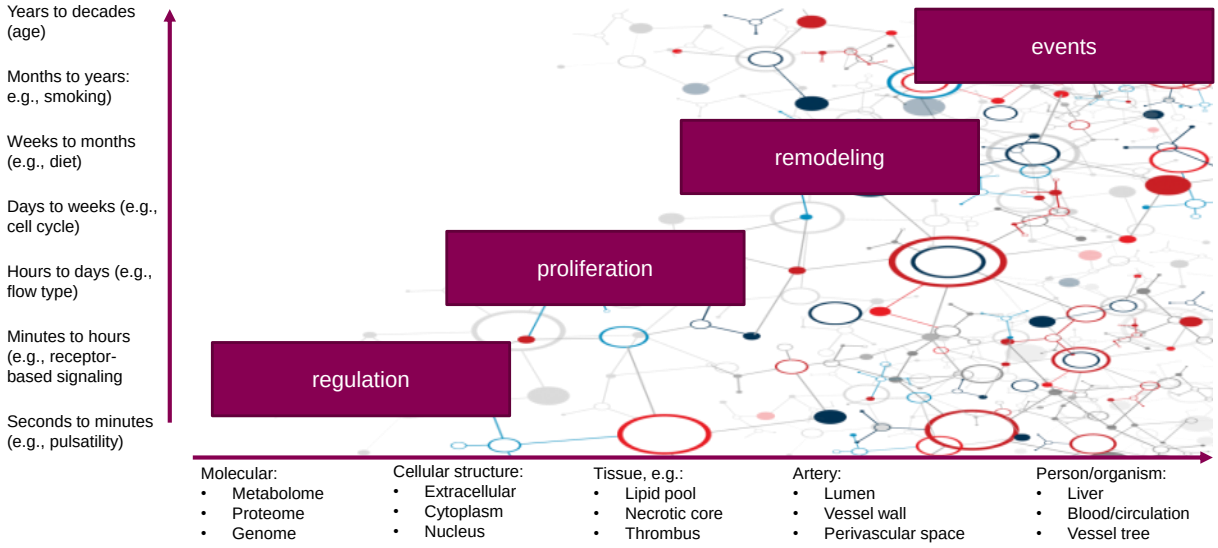


***Figure 6**: Temporal and spatial Scales that represent atherosclerosis*

Further outcome and cost improvements will flow from improved noninvasive diagnostics to identify which patients have progressing disease under first-line treatments. Based on the problems and opportunities in the field, computational modeling techniques may be applied to express relationships that span multiple time- and spatial- scales. In cancer research, the determination of molecular signatures of tumors using non-invasive imaging with biological information from tissue biopsies used as a truth basis has been met with enthusiasm [292-298]. Can this, or even something better, be achieved in vascular imaging?

3.1 PATIENT COHORTS

Transcriptomics studies in Studies 1 and 2 were conducted with 44 patients (40 development, four sequestered test) undergoing stroke-preventive CEA for high-grade (> 50% North American Symptomatic Carotid Endarterectomy Trial (NASCET) [299]) carotid stenosis. Patients with high vs. low calcified carotid lesions on CTA were selected as previously described [300]. Briefly, CEAs were collected at surgery and retained within the biobank (Biobank of Karolinska Endarterectomy, BiKE); patients without qualifying symptoms within six months prior to surgery were categorized as AS and indicated for carotid endarterectomy based on results from the Asymptomatic Carotid Surgery Trial (ACST) [301]. Patients with atrial fibrillation and those who had suffered a major stroke were excluded from the study. Carotid endarterectomies and peripheral blood samples were collected at surgery and retained within the BiKE. Details of sample collection and processing and transcriptomic analyses by Affymetrix microarrays were as previously described [302, 303]. Briefly, plaques were divided transversally at the most stenotic part; the proximal half of the lesion was used for ribonucleic acid (RNA) preparation; while the distal half was fixed in 4% formaldehyde and prepared for histology. The microarray dataset is available from Gene Expression Omnibus (GSE125771).

Symptomatology prediction in Study 1 was conducted by extending the transcriptomic study cohort by an additional 52 patients (overall n=96).

The biomechanical investigation in Study 3 was conducted as a case study. The case is a 67-year-old non-smoking man without previous medical history of stroke, diabetes, myocardial infarction, or peripheral arterial disease. The patient was referred to the vascular surgery outpatient clinic because of an asymptomatic 70-99% stenosis in the right internal carotid artery found on duplex ultrasound.

Protein to protein interactions were modeled in Study 4 based on data from patients on statin therapy undergoing stroke-preventive CEA. They were prospectively enrolled to represent the differences in protein levels between unstable and stable atherosclerosis. Specifically, 18 patients with CTA, histology, and plaque proteomics data for complete characterization (comprising three spatial scales). Three primary phenotypes were included: tissue from the center of the specimen representing stable or unstable plaque and from the periphery representing minimal disease. CEA specimens were processed for histology, and the global proteome was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) [304], yielding a total of 6735 proteins.

Supporting studies applied a subset of the primary study results to clinical applications:

- *CT angiographic biomarkers help identify vulnerable carotid artery plaque* [305] analyzed n=221 patients with asymptomatic carotid stenosis and at least three years of follow-up for major adverse neurovascular events.
- *Estimating the Precision of Quantitative Imaging Biomarkers without Test-Retest Studies* [306] analyzed n=31 endarterectomy patients.
- *Proteoglycan 4 Modulates Osteogenic Smooth Muscle Cell Differentiation during Vascular Remodeling and Intimal Calcification* [307] performed a cross-sectional study on carotid endarterectomies for which molecular assay was available.
- *Coronary plaque assessment of Vasodilative capacity by CT angiography effectively estimates fractional flow reserve* [308] analyzed n=113 patients with suspected CAD who had undergone CTA and invasive FFR.
- *Automated plaque analysis for the prognostication of major adverse cardiac events* [309] analyzed n=45 patients with suspected coronary artery disease, of which 16 (33%) experienced MACE within 12 months.
- *Association of S100A8/A9 with lipid-rich necrotic core and treatment with biologic therapy in patients with psoriasis: results from an observational cohort study* [310] analyzed n=125 psoriasis patients and *Treatment of Psoriasis with Biologic Therapy is Associated with Improvement of Coronary Artery Plaque Necrotic Core and Positive Remodeling: Results from a Prospective, Observational Pilot Study* [90] analyzed n=209 consecutive biologic naïve psoriasis patients, both cohort with coronary artery disease as a co-morbidity.

Supporting studies [311] and [312] review the state of the art in plaque morphology in neurovascular care on carotid artery analyses, and [282] in cardiovascular care based on coronary artery analyses, and [313] was an invited commentary rather than and not patient studies.

All patient imaging and samples were collected with Ethical Review Board approval and informed consent from patients.

3.2 HISTOCHEMISTRY

CEA specimens were fixed in 4% formaldehyde, and calcified plaques de-calcified in Modified Decalcification Solution (HL24150.1000). Specimens were rinsed, dehydrated, embedded in paraffin, and axially sectioned at selected distances from the bifurcation and the origin of the external carotid as guided by CTA and the location of defined plaque components in processed images. Sections were deparaffinized with Histolab clear (Histolab, Sweden) and rehydrated. Calcification was visualized with Alizarin Red (Sigma-Aldrich, Germany), followed by dehydration in acetone and acetone-xylene (1:1). IPH was detected by Perl´s Blue staining (Histolab, Sweden), rinsed with water, and counterstained in nuclear fast red. Intraplaque lipids were detected with Oil Red O (Sigma O1391). Slides were rinsed in 60% isopropanol and counterstained with HTX (Mayers, Sigma-Aldrich, Germany). For antigen retrieval, slides were subjected to high-pressure boiling in DIVA buffer (pH 6.0). After blocking with Background Sniper, anti-TGFBR2 (Abcam 186838; Cambridge, MA) and anti-IL1R1 (Abcam 106278) were diluted in Da Vinci Green solution, applied on slides, and incubated at room temperature for 1 hour. Isotype rabbit and mouse IgG were used as primary antibodies for negative controls. A probe-polymer system with alkaline phosphatase was applied, with subsequent detection using Warp Red. Slides were counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA), dehydrated, and mounted in Pertex (Histolab, Gothenburg, Sweden). Images were taken in an automated ScanScope slide scanner. All staining was performed according to the manufacturer's instructions.

3.3 MOLECULAR ANALYSES

Gene expression was analyzed by microarrays as previously described [300, 302]. In brief, RNA was extracted from CEAs in the transcriptomics cohort (n=44), and gene expression was analyzed in Affymetrix HTA 2.0 arrays in one batch (Affymetrix, Santa Clara, CA). Annotation was based on the Hg19 genome build, NCBI genome version GRCh37, and NetAffx build 34 for all 70523 probe sets. Signal space transformation and robust multiarray average normalization was performed on the Transcriptome Analysis Console software (Thermo Fisher Scientific), and processed gene expression data was returned in log2-scale. The microarray dataset is available from Gene Expression Omnibus (GSE125771).

LC-MS/MS analysis and protein identification was performed using methods previously described [304]. Plaques from selected patients were processed for proteomic analysis. Proteomic processing was performed using high-resolution isoelectric focusing (high-resolution isoelectric focusing, HiRIEF [314]) with median normalization of ratios on the

peptide spectrum match (PSM) level. Fourier transform mass spectrometry (FTMS) master scans were followed by data-dependent MS/MS. Spectra were searched using MSGF+ (v10072) [315] and Percolator (v2.08) [316], where search results were grouped for Percolator target/decoy analysis. PSMs found at 1% PSM- and peptide-level FDR (false discovery rate) were used to infer gene identities, and median normalization of ratios on the PSM level was performed. Protein level FDRs were calculated using the picked-FDR method [317].

3.4 QUANTITATIVE CTA ANALYSIS

Increasing evidence shows that CTA (both carotid and coronary) may be an ideal modality to fill gaps in understanding the extent and rate of progression of atherosclerosis [318-321]. Advanced software-based techniques to extract data embedded in images, which are otherwise not readily appreciated visually or quantitatively, are being implemented to augment subjective assessment. Quantitative CTA analysis has the potential to move from subjective and qualitative to objective and quantitative analyses as needed for the formal assessment of biomarkers [322]. The utility of CTA in detecting and managing both obstructive and non-obstructive atherosclerotic lesions is increasingly documenting drug treatment effects [200, 323]. Recent drug trials provide potential plaque biomarkers to demonstrate the efficacy of new medical therapies [324, 325]. CTA utilization is increasing due to solid outcome study results that show better performance and at a cost less than functional testing [326-328].

In particular, ElucidVivo (Elucid Bioimaging Inc.) was developed to overcome two fundamental issues that have previously limited quantitative performance. ElucidVivo adopts histopathology as the performance standard of ground truth basis to allow specific tissue labeling approved for clinical use by regulatory bodies such as the FDA [163, 309, 329-337]. ElucidVivo characterizes tissues utilizing a histologically validated algorithm to quantify CALC, LRNC, and IPH accurately. Figure 7 shows measurands, and Figure 8 examples of concordance with histopathology. The software achieves an effective resolution approximately 3x higher than the reconstructed voxel size with improved soft tissue plaque component differentiation relative to manual inspection. This is achieved using an adaptively determined, patient-specific, 3D point spread function with the effect that image intensities are restored to more closely represent the original materials imaged. This approach mitigates artifacts such as calcium blooming, enabling less visually conspicuous tissue types to be delineated [338, 339].

Analytic performance of ElucidVivo has been undertaken for tissue composition accuracy relative to histopathology [331], and reader repeatability and reproducibility have been determined [330]. Performance characteristics are included in the commercially available clinical edition software regulated as a medical device [340]. ElucidVivo's approach is an ideal platform for developing multivariable and multivariate models. Interpretable, rather than only black-box, models for phenotype classification and risk stratification are thereby enabled. Applications in carotid disease [311, 312] and coronary disease [90, 282, 309, 341] are increasingly reported.

Multiple objectively validated measurements may be made to characterize plaque morphology. Plaque morphology comprises structural anatomy and tissue composition (Figure 7). Measurements may be performed in 3D and from the 3D, at cross sections at various spacing (e.g., 0.5mm). The absolute volume (per artery, per lesion, cross-section), the proportional occupancy, and maximum values for each artery and lesion are calculated. Wall area or volume may be calculated as the overall vessel volume or area minus the lumen area or volume. Plaque burden is assessed as the ratio of wall area and volume divided by the overall vessel area or volume. Lesion length is calculated using the centerline in 3D. Volume calculations are determined from 3D regions at the overall target and marked lesion levels. Vessel structure measurements may include, for example, the degree of stenosis (calculated both by area or diameter), wall thickness (e.g., the distance between the lumen boundary to the outer vessel wall boundary), and remodeling index (e.g., the ratio of vessel area with plaque to a vessel area without plaque used as reference). Measurements for tissue characteristics may include, for example, calcification, lipid-rich necrotic core plaque, intra-plaque hemorrhage, and matrix/fibrous tissue, each with volume and cross-sectional area measurements and proportional occupancy.

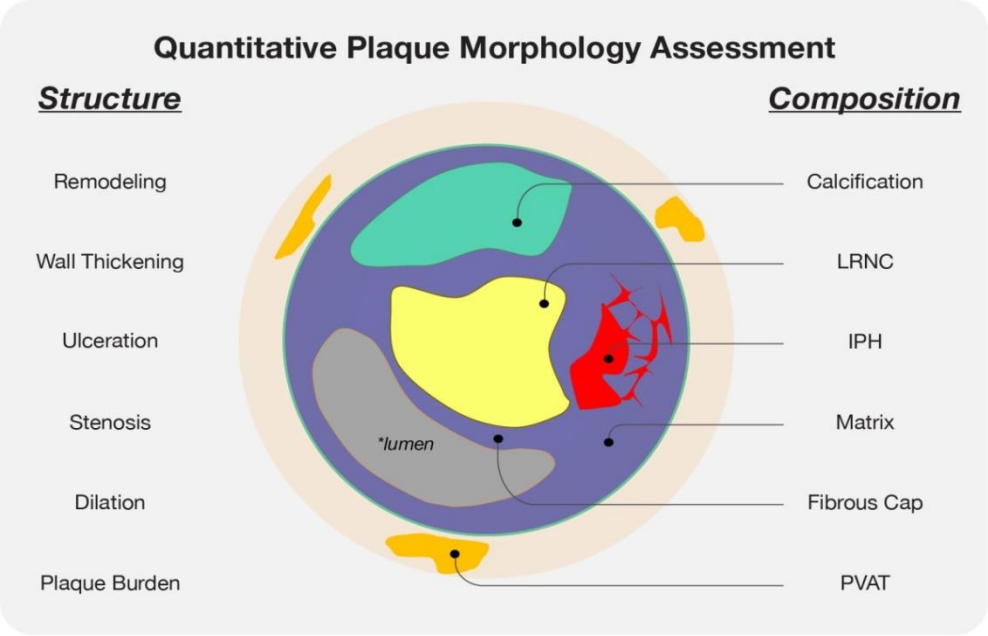


***Figure 7**: Multiple objectively validated measurements to characterize plaque morphology by CTA analysis software. These assessments included structural anatomy ("structure") and tissue characterization ("composition"). (LRNC=lipid-rich necrotic core, IPH=intra-plaque hemorrhage, PVAT=perivascular adipose tissue). Not shown but also detected may be micro-calcification, or mural or white thrombus. Also assessed but not well represented by the figure are fissure and ulceration.*

Ground truth data may exist at different spatial scales, such as the tissue scale (histopathology) or the molecular scale (RNA transcripts, proteomics, metabolomics, etc.). Figure 8 shows the processing of CTA demonstrating various tissue types, each with corresponding histopathology annotation. The annotation may be done with multiple stains, for example, Hematoxylin, Perl's

blue, Masson's Trichrome, or Movat. For example, histological sections may be taken from endarterectomy or autopsy specimens (Figure 8).

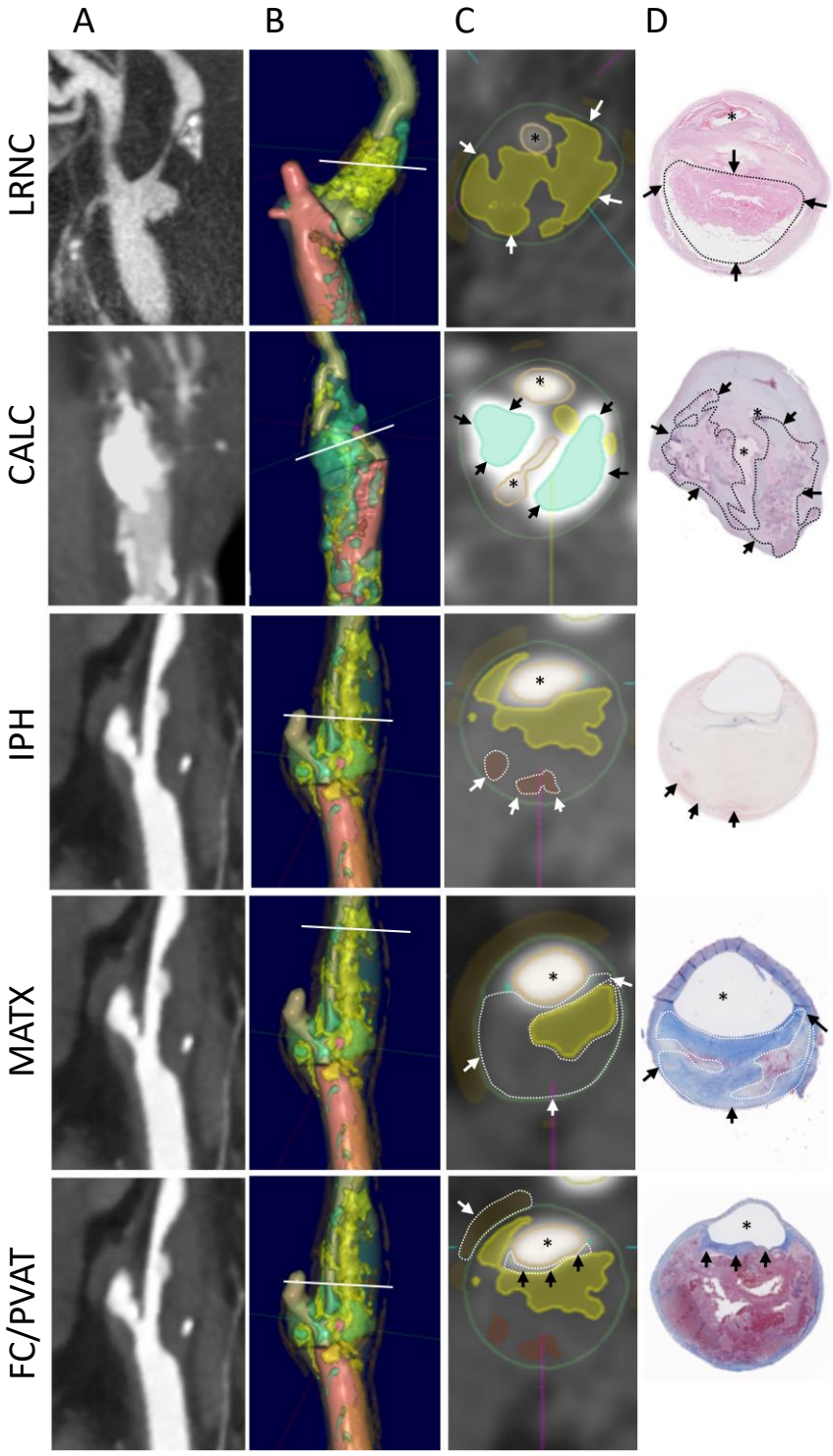


***Figure 8**: Processing of carotid CTA (A) with approach demonstrating 3D image (B), axial view of plaque (C; white line in B indicates the position of section; yellow LRNC) and corresponding histological section (D) stained with Hematoxylin (LRNC, CALC), Perl's blue (IPH; arrows) and Masson's Trichrome to visualize fibrous tissue (MATX). * signifies the lumen; dashed lines and arrows mark tissue components. FC is the fibrous cap (arrows between LRNC and lumen). PVAT outlined (C; white arrow) is not included in the histological sections of endarterectomy specimens.*

Molecular-scale ground truth comprises genomic, transcriptomic, proteomic, and metabolomics. For example, gene expression may be analyzed by microarrays, RNA may be extracted from tissue specimens, and gene expression analyzed using arrays with conventional or single cell techniques. Various forms of mass spectrometry are used for proteomics or metabolomics. Bioinformatics processing steps may be applied, such as signal space transformation.

3.4.1 Applicability of carotid specimens for use across arterial beds

Given that we used carotid artery specimens harvested in endarterectomy procedures as a representative artery, it may be questioned whether image recognition validated against carotid tissue applies to plaque in other vascular beds, particularly the coronary. This is important because tissue collected from live patients with contemporaneous CTA is superior to autopsy specimens with a longer time difference in when the CTA is performed, and the tissue changes post-mortem that would otherwise result in lower quality comparisons. As such, the carotid artery would be considered not only an acceptable but even an ideal model artery, provided those characteristics utilized by the analytical technique are optimized to exploit the commonalities while mitigating the difference across beds:

1. First, technical factors such as how superficial the arteries are and motion are accounted for in accepted protocols, having been optimized for human interpretation and which our algorithm uses to its advantage.
2. Second, while it is true that the frequency and relative contribution of plaque progression, rupture vs. erosion, and thrombosis may vary in coronary compared with the carotid arteries and other arterial beds, the tissues on which these processes act share common definitions:
    - Plaque characteristics such as a large atheromatous lipid-rich core, thin fibrous cap, outward remodeling, infiltration of the plaque with macrophages and lymphocytes, and thinning of the media predispose to thrombosis in both carotid and coronary artery disease [99].
    - Tissues in the carotid and coronary arteries have many similarities in the physiology of vascular tone regulation that affects plaque evolution [99].
    - Blood perfusion in the distal myocardial tissue is regulated by the vasodilation of proximal coronary arteries in response to various stimuli such as nitric oxide, causing dynamic changes in blood flow. Similarly, carotid arteries are more than simple conduits supporting brain circulation; they demonstrate vasoreactivity in response to stimuli, including shear stress changes [342].
    - Endothelial shear stress contributes to the vascular wall transcriptomic profile [232]. Clinical studies in both the coronary [100] and the carotid arteries [101] identify lower wall shear stress associated with plaque development and localization according to a common mechanism.

    The above points support the conclusion that whereas the extent of the various plaque tissues differs across arterial beds, the cellular and molecular level milieu of the

individual tissue types share common objective definitions across beds [98]. Specifically, whereas inter-bed differences such as a thicker fibrous cap and a higher prevalence of intra-plaque hemorrhage in carotid vs. the coronaries affect the amount of these tissues [95], it does not change the nature of the tissues when they do present. It is the cellular and molecular level milieu on which the algorithm operates, not the prevalence, enabling our choice of tissue model for tissue characterization.

3. Third, clinical differences in risk and disease manifestation across beds are accounted for by using additional factors explicitly incorporated into our approach that go beyond the tissue characterization. These include, for example, the use of sophisticated normalization techniques for vessel size, plaque geometry and rheology, collateral flow, and the downstream circulations (myocardium vs. brain) to augment the tissue characterization algorithm itself.

3.5 MULTI-SCALE MATHEMATICAL FORMULATION AND PREDICTIVE MODELING

Figure 9 shows one manifestation in terms of a mathematical framework for multi-scale analysis. The expression *y*(t) refers to a phenotype *y* at time t.

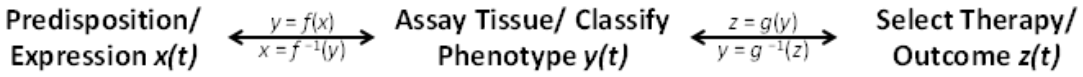


***Figure 9**: Mathematical framework for multi-scale analysis.*

The expression *x*(t) designates cellular and molecular levels at time t, and *z* represents the patient-level outcome or state at time t. The approach is to determine systems of equations, or non-linear models, *f* and *g*, where *f* goes down in scale and *g* goes up in scale. For example, Study 2 approached the function *f* in a predictive modeling paradigm, with *y* being expressed as a vector of scalar values (but may be extended in future work to operate on multidimensional data) to derive expression profiles. Study 4 extends this to protein and metabolite levels. One manifestation for function *g* may also be a predictive model, but a different model than *f*, one that moves up in scale. Inverse functions for *f* and *g* may also be derived.

Steps may be outlined for determining the function f (Figure 10). Training data sets are used to optimize models of various types, and the results may be further applied o supervised or unsupervised clustering. The resulting associations, at the cohort or individual level, may be analyzed using techniques such as gene-set enrichment analysis (GSEA) to elucidate biological processes and molecular pathways at the cohort level or in individual patients. GSEA may be conducted, for example, using EnrichR (https://amp.pharm.mssm.edu/ Enrichr/), further passing results from the Gene Ontology Biological process, and further by analyzing data with other systems such as Revigo (http://revigo.irb.hr/) to determine, for example, non-duplicative processes. Individual patient-level inference may be applied where both the degree of dysregulation and the statistical model significance may be considered. This may be variously described as Virtual Transcriptomics, Virtual Proteomics, or Virtual Metabolomics.

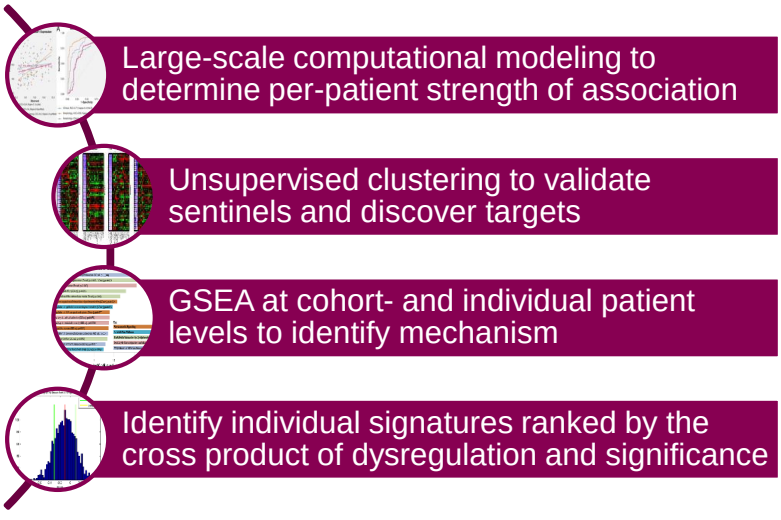


Figure 10: Training and inference in virtual transcriptomics

The Virtual Transcriptomics, Virtual Proteomics, or Virtual Metabolomics models themselves may be performed, without loss of generality, for example, by the following. All or a selection of probes from a microarray, species from mass spectrometry, or other assay methods for 'omic data, may be selected. Single and multiple variable regression models, covering linear and non-linear modeling techniques, may be performed on predictor sets drawn from heterogeneous data. Predictors may be drawn from plaque morphology, demographics, clinical (laboratory) values, or other variables. This is done in part to recognize that a diversity of variables could have affected the results, to inspect the added value of given variables, and to identify when variables have independent information content.

Each model result may be output and tabulated to identify the highest-achieved performance on a species-by-species basis. Predictive performance may be determined based on the accuracy of the prediction relative to the true or reference values. Models may be built with variations, for example, differing sets of morphological measurements according to hypothesized physiological rationale; automated optimization using, for example, cross-validation while simultaneously varying tuning parameter values; or partitioning data such that a training set on which the cross-validation was performed was strictly separated from a sequestered validation data set to test performance using locked-down models. The use of histologically validated plaque features, for example, may produce interpretable models [343] and, when coupled with cross-validation, may mitigate overfitting.

Supervised model quality (MQ) was determined as the product of two measures for each model type. MQ for continuous estimation models was computed as the product of concordance correlation coefficient (CCC) and regression slope of predicted vs. observed for continuous value estimation (the former to measure the tightness of fit but augmented by the latter to ensure proportional prediction relative to observed). MQ for dichotomized categoric prediction models was computed as the product of area under the receiver characteristic curve (AUC) times Kappa for dichotomized prediction. AUC to measure the net classification performance but augmented by Kappa to ensure performance in both high and low expression classes.

The traditional models identify the differences across groups but are not generally applicable on a per-patient basis. They do not generally account for "overfitting" or consideration for accuracy on an individual patient basis, as do machine learning techniques [344]. Deep learning neural networks extend this for use on higher dimensionality data sets such as 2D and 3D images. The quality of a model is only partially represented by its performance metrics; models that are likely to extrapolate well to unseen patients based on a documented rationale may be described as "interpretable" models [343]. Model interpretability is improved by using histologically validated biomarkers as inputs and being architected according to a rigorously understood and biologically plausible mechanism.

3.5.1 Per-patient Linear and Logistic Regression

Predictive models may be used to quantify the degree to which variables are associated. Linear regression models take the form:

$$\hat{y} = mX + b \quad (2)$$

where response variable Y is estimated as ŷ, being a linear combination of one or more predictor variables X plus a constant. The related logistic regression is used when Y is a categoric rather than a continuous response variable (usually via a probability output). Feature "selection" identifies what to include in the model (the Xs).

3.5.2 Nonlinear Models

Feature "engineering" can be done to provide various mathematical combinations or processing prior to use in the regression equation that incorporates domain knowledge explicitly to aid model fit. The transformation functions may not be linear, or the decision theoretic used may be of a form other than linear to capture more complex relationships. Non-linear models take the form:

$$\hat{y} = f(X, \beta) + \varepsilon \quad (3)$$

where the function f may take forms other than linear combination. There are many approaches to constructing the function f, including, for example, support vector machines (SVMs), tree-based models, and neural networks. Like their linear counterparts, these model types generally use vectors of scalar inputs.

A range of model types was used in this work as, in general, we did not know which type would best fit the data *a priori*. An example of a non-linear function is an artificial neural network, which may be expressed as interconnected linear combinations of the inputs but organized in layers, where one layer "activates" the next layer using an "activation" function ψ:

$$z = \sum_{k=0}^{n} w^k x^k + b \quad (4)$$

$$a = \psi(z) \quad (5)$$

where ψ may be biologically inspired, such as a sigmoid, and ŷ is taken from the last layer.

Feature selection in artificial neural networks occurs by optimizing the value of coefficients applied on measurements to "hidden" units and then from these hidden units to the output nodes, which express the output as class probabilities (e.g., avNNet [345]). Another model type that often performs well is least squares regression models with a form of regularization to optimize the tradeoff between bias and variance called ridge regression [346]. Optimization in these models occurs by determining iterating over values of λ, where low values favor low bias to higher values that allow successively higher values of bias to reduce variance.

The actual model development process proceeds with three levels of variation: first, by using different sets of predictors according to the hypothesized physiological rationale confirmed by the unsupervised clustering; second, for each set, automated optimization using bootstrap or repeated k-fold cross-validation while simultaneously varying different tuning parameter values appropriate to each model type; and third, application to strictly sequestered hold-out sets.

3.5.3 Deep Learning Models

Model types with higher dimensional input data allow for these models to retain the spatial context of, for example, distributions of tissue types, rather than being limited only to their magnitude:

$$z = \sum_{j=0}^{n} \quad \sum_{k=0}^{m} w^{j,k} x^{j,k} + b \qquad (6)$$

and activation functions being as previously described.

Methods described as deep learning, particularly when input data are first processed using convolutional layers followed by fully connected layers are presently attracting attention. These have become popular based on computer vision applications such as driverless cars, facial recognition, satellite imagery, etc. Medical applications include object detection, semantic segmentation, and phenotype classification. Still, there are two significant drawbacks to be mitigated: 1) the amount of annotated training data (scaled to cover all input variability) and 2) interpretability, sometimes referred to as the "black box" problem. Both conspire against success in medical applications, resulting in overfitting or lack of generalization, largely because there is no possibility to generate sets as large as used in other computer vision applications due to cost and privacy issues. In this work, we mitigate the two drawbacks but keep the benefit of the nonlinearity of the network architecture.

3.6 BIOMECHANICAL ANALYSES

Signed distance function data files acquired with ElucidVivo were converted into geometry objects (iso2mesh [284] and Matlab (MathWorks, Natick, MA)) that allow for structural and hemodynamic analysis with the Finite Element Method (FEM). This generated triangular surface meshes of all morphological entities. Morphological processing and smoothing were used to ensure that the wall domain always surrounds the lumen domain. The resulting spatial representation of the diseased vessel was saved in the standard triangle language (STL) format and imported into FEniCS-HPC [347] for hemodynamic analyses and into COMSOL Multiphysics® software (COMSOL AB, Stockholm, Sweden) for structural wall analysis. Minor manual adjustments were made to create clear inlet, and outlet faces for boundary condition application.

3.6.1 Hemodynamics

3D ultrasound Doppler acquisitions were used to measure the blood flow velocity over the cardiac cycle. This data was filtered and used to identify the flow at the inlet. CFD analysis was used to explore the hemodynamics in the artery in terms of the spatial and temporal distribution of the blood pressure acting on the vessel wall. Given the complex flow in diseased arteries, the Carreau model described the shear-rate-dependent viscosity of blood using parameters extracted from the literature [104, 120]. The Carreau model was implemented as a discontinuous constant field, making the problem locally Newtonian, and solved as a Navier-Stokes problem with variable viscosity.

3.6.2 Wall Analyses

Structural biomechanical models used homogeneous as well as inhomogeneous representations of vessel wall tissue, according to an incompressible Yeoh strain energy function

$$\psi = \sum_{i=1}^{3} c_i (I_1 - 3)^i \qquad (1)$$

to describe the vascular tissues [348]. Here, $c_i; i = 1,2,3$ denote stress-like material parameters, whilst I_1 is the first strain invariant.

In the homogeneous structural model, the Yeoh parameters were constant over the vessel wall, while a linear 3D interpolation function prescribed the parameters in the inhomogeneous model. The individual parameters within the respective tissue composition were acquired by the *in vivo* tissue characterization at the spatial locations determined by ElucidVivo. The finite element mesh did not have to strictly abide by region boundaries to obtain an effective mesh while retaining spatial heterogeneity. A fine mesh was utilized to adequately capture the transition of the material properties at composition interfaces. The prescribed structural tissue properties were based on published literature [124].

3.7 SYSTEMS BIOLOGY MODELS OF MOLECULAR PATHWAYS

The models mentioned above capture associative but may not capture causal relationships between the predictors and the response. A formalism representing biological signaling pathways needs to be incorporated to model causality. Such models are often described as

"systems biology" models. A review of approaches to *in silico* systems biological models was undertaken to identify the most promising current strategies across disease areas [349-359] and apply them to atherosclerosis (Figure 11).

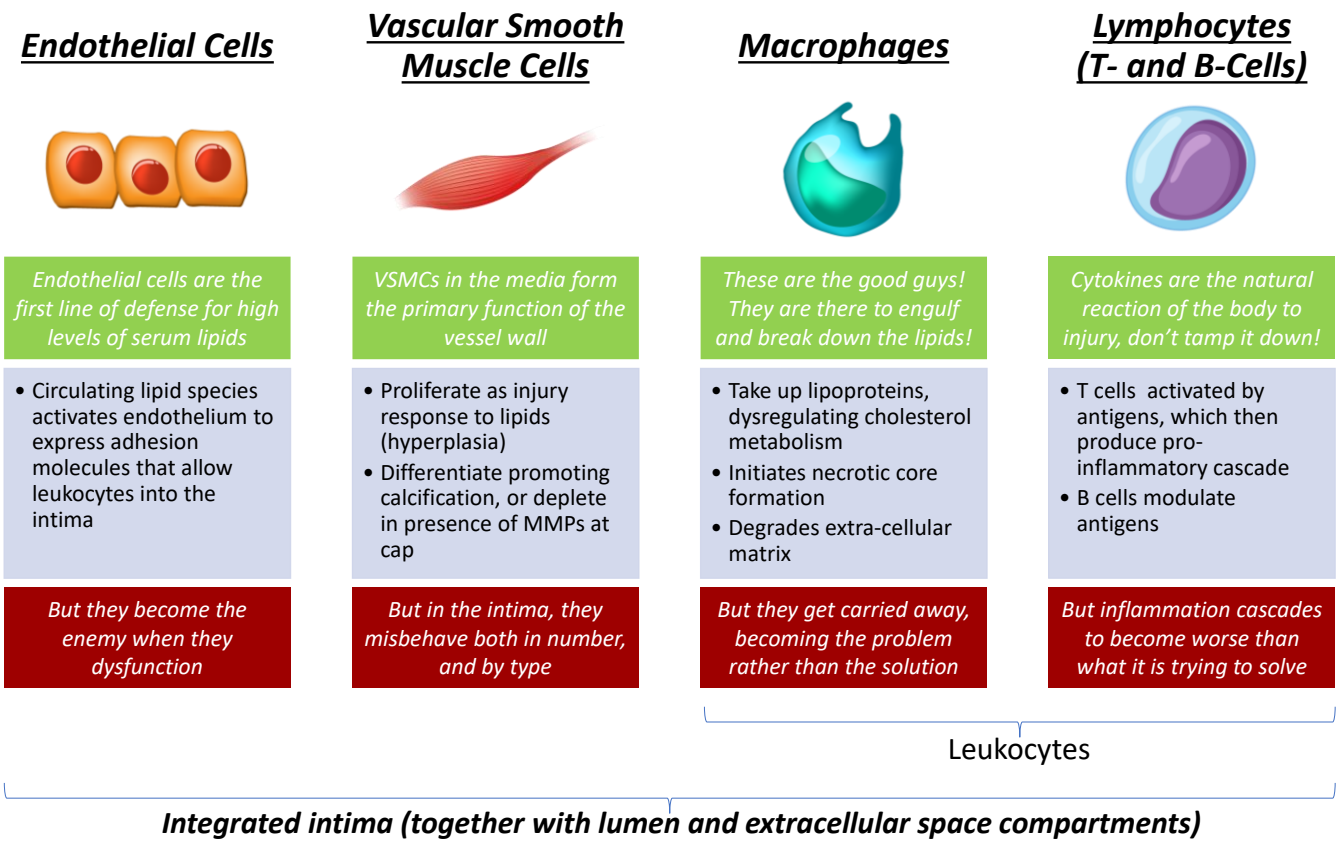


Figure 11: Primary components necessary for a clinically relevant systems biology model of atherosclerosis

3.7.1 Quantitative Systems Biology Models

Protein-protein interactions in quantitative systems biology models may be built using "ordinary" differential equations (ODEs) interconnected with networks. The equations characterize how the change in an independent variable changes the value of a dependent value. This is represented by using differentiable functions:

$$a_0(x)y + a_1(x)y' + a_2(x)y'' + \ldots + a_n(x)y^{(n)} + b(x) = 0 \quad (7)$$

where $a_0(x)$, ... , $a_n(x)$and $b(x)$are defined functions that represent an empirically observed or hypothesized relationship in terms of change between the predictor variables x and the response variable y. The functions a do not need to be linear, but they must be differentiable. Whereas some differential equations may be solved explicitly, in general, it is necessary to use numerical methods that approximate rather than solve for the solution. Typical functions include mass action functions that express the rates of chemical or biological reactions/relations in terms of how products are produced from reactants. By way of example, Michaelis–Menten kinetics, a specialized formulation of how enzymes function; equations governing inhibition are used to express how the presence of species may cause reduced rates of production of products, as are typical of biological signaling pathways.

The term “ordinary” refers to the bulk consistency of the relationship without regard for spatial or time variation. That is, the relationship is the same anywhere in the postulated space, and the relationship doesn’t change with time. Generally, this is applied to “compartments,” localized spatial regions or periods of time where these constraints are approximately true. These are particularly important examples of biochemical (compartment) models used in pharmacokinetic and pharmacodynamics studies.

More advanced models to capture increasingly complex interdependencies that account for either spatial or temporal variant behavior are represented by systems of “partial” differential equations (PDEs) that generalize on ODEs using partial derivatives that allow for the spatial or temporal variation. If the granularity of the process is sufficiently modeled in relatively coarse “compartments,” e.g., like what happens in the intima vs. the liver, ODEs suffice. If, on the other hand, what happens inside of a compartment is of consequence, e.g., the endothelial surfaces vs. migration into the media, either the compartment must be decomposed into smaller compartments, or if gradients or continuous variation rather than discrete variation is of interest, then PDEs are needed. If structural relationships of biomechanical forces among entities (e.g., between cells, proteins, etc.), formalisms such as CPM models may be used in conjunction with systems biology models.

Mathematical formalisms for extending multi-scale models to identify causality explicitly have been explored [349-356], such as intracellular cell cycle networks (e.g., by ODEs), regulation of the collective behavior of cellular structures (e.g., CPM), spatially differentiated behavior (by PDEs), which in turn control intracellular behavior (returning to ODEs). Proposed model creation and validation workflows [357-365] rely on high-level hypothesis generation and then the collection of data. Whereas data has been documented at individual scales, i.e., *in vitro* data at the cellular and molecular scale, microscopy data at the histopathology scale, and radiological data at the macroscopic scale, there is a dearth of linkages across these scales.

3.7.2 Semi-quantitative Systems Biology Models

Attempting clinical-scale models with complete reaction kinetics suffers from three primary challenges. First, attempting to comprehensively capture all protein-to-protein interactions, much less all intermediate products in the metabolome, is generally beyond the existing scientific knowledge and, in any case, would be an overwhelming task. Second, even if undertaken, were it to be done by a single group, there would be a general lack of peer review or public adjudication on the biology, exacerbated by the first challenge but a problem of its own. Third, even if large swathes of the interactions were to overcome the first two challenges, even the smallest gaps would invalidate the model as the quantitative simulations do not tolerate “gaps” or inaccuracies well. A critical insight is that accomplishment of the objective does not require fully quantitative models; rather, semi-quantitative or even qualitative models would identify the “direction” of drug response even if not the exact strength of that response. This observation is broadly recognized [366-369].

Qualitative (or semi-quantitative) models formulated based on Boolean algebra or other mathematics [370-373] that fall short of complete reaction kinetics still identify directionality, or even levels of magnitude, to satisfy our requirements. The use of "relations" in place of "reactions" over a portion or even the whole model simultaneously solves the brittleness problem while also allowing the use of publicly peer-reviewed pathways such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [374, 375]. Our work establishes the feasibility of using publicly curated systems of pathways with the level of completeness needed for clinical use rather than only small-scale demonstrations [376-378]. Moreover, provided programming was undertaken to update from the publicly available updates through such resources continually, would allow the model to evolve as the scholarship evolves.

3.8 BIOINFORMATIC AND STATISTICAL ANALYSES

Several bioinformatic and statistical analyses were used in support of the thesis.

Analyses of differential gene expression were performed using the volume proportions of components and structural categories. Gene expression profiles of plaques with the highest measured values were compared with those with the lowest to optimize the identification of biological processes associated with either of the categories and to mitigate the inherent heterogeneity in plaque morphology (10 vs. 10 for components and 5 vs. 5 for structural category). Groups were analyzed using limma [379] with empirical Bayes moderation of the standard errors towards a global value, implemented in Transcriptome Analysis Console (Thermo Fisher Scientific).

Significantly differentially expressed genes and ontologies were identified with FDR $q<.05$, and differentially expressed genes were displayed in volcano plots. For the identification of biological pathways, ranked lists of genes according to p-value were processed through gene set enrichment analysis [380] (v. 4.1.0; Gene Ontology (GO) Biological Processes c5.go.bp.v7.2.symbols.gmt) and then run through Cytoscape (v.3.8.2) with Java (v.11.0.6) where the cut-off p-value and FDR q-value were both set to $<.005$ to visualize top (up-regulated) and bottom (down-regulated) ontologies and pathways. Clusters were created with the AutoAnnotate function as previously described [381].

Correlations between different types of plaque morphology (anatomic structure and tissue characteristics) were performed with univariate analysis using GraphPad Prism 8 correlation matrix and Pearson's coefficient, and correlogram using R (package corrplot2017, v 0.84). The association between symptomatology and plaque morphology was explored with Spearman's coefficient and a two-sided Student's t-test, assuming normal distribution and equal standard deviation. Variables not fulfilling the criteria for normal distribution (e.g., MinCapThickness) were log-transformed into normality before the t-test.

Unsupervised clustering provided a rough sense of relationships between plaque morphology and expression levels. The hierarchical clustering is represented as a dendrogram split at points with a Pearson correlation less than 0.8 using a Euclidean distance function according to the complete linkage method on both plaque morphology measurement features and expression

levels, plotted as a heatmap. Supervised MQ was determined as the product of two measures for each model type. MQ for continuous estimation models was computed as the product of CCC and regression slope of predicted vs. observed for continuous value estimation (the former to measure the tightness of fit but augmented by the latter to ensure proportional prediction relative to observed). MQ for dichotomized categoric prediction models was computed as the product of AUC times Kappa for dichotomized prediction (the former to measure the net classification performance but augmented by the latter to ensure performance in both high and low expression classes).

Predictive models were fixed ("locked down") and applied to sequestered patients selected at random for which ground truth was known to validate the performance of the model on patients not included in the development of the model ("unseen patients") to test generalizability [344]. For each test patient, we used the models for transcripts that were particularly robustly predicted and determined the significance of the predictions by applying a bootstrap method to permute plaque morphology inputs to each transcript's model, providing a measure of model stability used to adjust the outputs for each test patient. Patient-specific GSEAs were determined from transcript ranking, and p-values for the process were adjusted for multiple hypothesis testing.

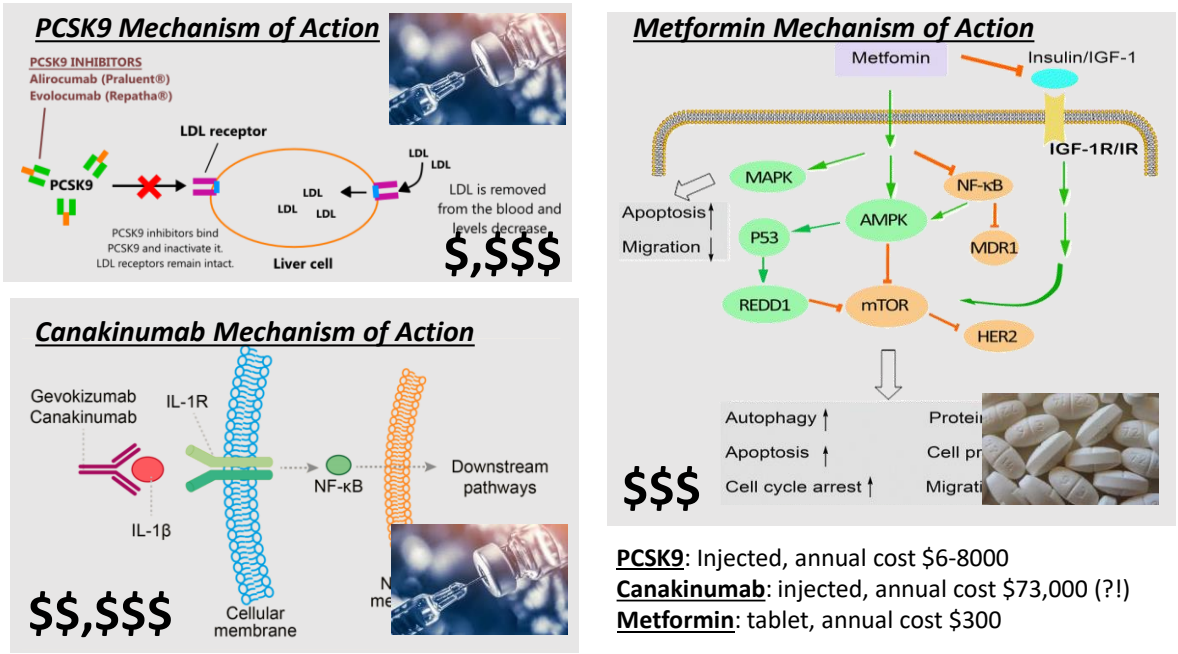
Demographic variables were summarized to characterize the cohort and identify significantly different values across plaque subgroups. Categoric variables with less than 25% missing data were tabulated with fractions, and significance was analyzed with Fisher Exact test. Continuous variables were tabulated as medians with inter-quartile range and significance analyzed by the Wilcoxon non-parametric test (using a confidence level of p=0.05).

Proteomics signatures were scored for instability using a rank-weighted combination and cosine correlation as a similarity metric from the exemplar database. For each patient, baseline and each simulated drug treatment, as well as a hypothetical drug combination, intensive lipid-lowering, and metformin, were modeled using multi-level statistical analysis (implemented in R using the "lme4" package). Explanatory variables of cell type, network scope, and interactions were included in the models. Subject level effects determined from posterior variances were used at both absolute and relative drug effects. This analysis method allowed subject-level values annotated with 95% confidence intervals and p-values representing the strength of the effect as a hypothesis test against the null hypothesis of no effect, i.e., no change in instability. Individual subject recommendations were expressed in terms of the relative improvement for each drug together with heatmaps showing protein levels for baseline conditions and after best treatment.

3.9 INTEGRATED APPROACH

Clinical decision support systems (CDSS) incorporating results from this thesis can be used for selecting and recommending a suitable treatment plan for a patient with cardiovascular disease to analyze and process non-invasively obtained data of arteries from patients with atherosclerosis to obtain predicted proteomic and genomic information. Based on this

information, various potential therapies (pharmacotherapies or procedural interventions) can be simulated based on their mechanisms of action in *in silico* systems biology models to provide a report for use by the health care provider recommending one or more specific therapy recommendations (Figure 12). This report may also be used directly with the patient to engage them in their care.



PCSK9: Injected, annual cost $6-8000
Canakinumab: injected, annual cost $73,000 (?!)
Metformin: tablet, annual cost $300

Figure 12: Many therapeutic alternatives exist, but the differences in mechanism and cost vary widely. Here just a few are shown to represent the range that are not currently capable of being prescribed broadly using only population-based approaches.

In silico systems biology model(s) are initially generated or trained with two data types. First, one uses experimentally determined data from biological specimens from development subjects. Development subjects are patients for whom actual proteomic data is available that shows differentially expressed 'omic levels that are linked to the specific characteristics and morphology of the plaques in each of those subjects. Second, one uses results from searches of public literature, experimental results, or other databases to create the initial *in silico* systems biology model.

The initial *in silico* systems biology model is then updated with calibration data from test subjects to validate and refine the initial model. The calibration data is again based on actual biological samples that show differentially expressed protein or transcription levels linked to the specific characteristics and morphology of the plaques in each test subject. This step confirms that the model works as intended and augments and renders the model more robust, given the calibration data from many test subjects.

In operation, the calibrated *in silico* systems biology model is again updated, but now with patient-specific personalized data based on imaging of clinical patients without the need to perform invasive procedures. The calibrated *in silico* systems biology model is perturbed for

potential therapy mechanisms of action. The effect is simulated to provide therapy recommendations based upon an automated comparison of the two or more different therapies whose predicted effects are programmed into the model.

3.10 ETHICAL CONSIDERATIONS

This research seeks to integrate histological, molecular, and biomechanics aspects of the diseased blood vessels in computational models of increasing complexity. Modeling activities were guided by established landmark research augmented by *in vitro* work and included state-of-the-art algorithms performing tasks, such as evaluating, searching, and filtering in parameter space; extraction of robust mechanistic hypotheses from the parameter sets (analyzing behaviors in state-space with perturbations of interest); validation (for establishing feasibility/accuracy of state variables in terms of model parameters. Given the number of species and specimens needed for deriving robust results, supercomputing resources with extensive data sets were utilized.

Ethical Considerations Unique to this Work

Building, evaluating, and reporting computational models is intrinsically fraught with potential ethical concerns because, by its nature, it includes feature selection and application of candidate models leading to either better or worse fits of the input data. However, it is hard to perform feature selection and engineering in an unbiased way; by definition, the exercise is to improve model fit, and the problem of overfitting is ubiquitous. Frequently, even the researcher, let alone reviewers or those who seek to understand what has been done, can tell if it is present. This causes at least two types of difficulty, unfortunately generally opposite errors. The first is that there is a certain degree of "hype" in the sense of what AI can do, and many may jump to the conclusion that the technology can solve many problems beyond what it could actually do or not be aware of the issues caused inadvertently even for successful applications. On the other hand, people can have a sense of disbelief in all models. Even if they do not entirely understand why they may have seen mistakes made or have not been convinced of good performance before and as a result, treat all new results with skepticism.

Questions for Reflection

If an outsider were to be designated a critical ethics reviewer for your research and had a strong incentive to find something to complain about, what would they likely focus on? The ethical dilemma is that not all data can be presented, given that these projects have many large data sets with very large amounts of intermediate results. Even if one thought it most ethically "clean" to present it all, they never could because the people who would use the models neither have the time or inclination to consider it all. Consequently, summaries need to be made, and only a subset of what was considered can ever be presented or published, leaving one with choices on what to include. Whereas it is said that negative results are as important to publish as positive results, the very definition of model optimization selects for positive results: what didn't work may have the most significant insights or demonstrate caveats and weaknesses, but it is intentionally and mathematically selected out. It is at the researcher's discretion that how much of the poorer results get represented and explained is inherently a matter of judgment that requires effort. Not everyone is equally scrupulous on this, and inducements to only demonstrate the positive part of the story are compelling, often existential. If one held the view

that they would be a positive example, presenting all the negative with the positive is not ideal either, as this is often just unhelpful. When people need to see what may be done, it isn't a virtue to pontificate on all the downsides because it turns the work into a morass and leaves the interpretation to others with just a sense of confusion presented: hardly virtuous.

How would you defend what you do? Using justified data methods, including feature selection methods backed by physiologically plausible justifications, is imperative. Whereas it is acceptable to represent a given result positively, believing that that is what offers value. Yet, limitations must be stated openly and include the degree to which the base of data contributed to it, the range of options considered, the optimization metrics used, and how they were applied. Additionally, in conducting peer review for journals, the peer reviewer must also seek this in others' work, which is less present than expected. Hopefully, the field will be continuously improved through critical reviews to provide the best service to the applications we seek to serve.

Is investing in the research you're devoting yourself to the proper priority, or would it be better to put resources into something else? Computational models are arguably among the most responsible activities when performed according to sound scientific practices, as it maximizes data use and reduces the need for new data collection. However, careful attention to generalizability to target populations is essential, and adequate sampling often requires expanding the data sets.

What consequences could your research have? Can the results, or the methods developed, be abused in any way? Is there a risk of any group being stigmatized? Is there a risk that another group with a greater need will have to step back for efforts to be made in your area? One particularly controversial topic is that the original informed consents anticipate broad uses of the data; otherwise, it may not have been clear to subjects how far their data would be "stretched." An example was the use of genetic data collected from American Indians to identify origins that inadvertently and unexpectedly violated their sense of originating in the Americas as opposed to migrating from Asia.

How is informed consent handled, why, and how is confidentiality and data storage, including sensitive personal data, handled? Whereas the typical application of informed content certainly applies to machine intelligence, a category of "exempt" research using data for retrospective analyses using anonymized data sets is common. Proper application of this category requires that the original data collection allows for it and that protected health information remains appropriately handled.

4 RESULTS AND DISCUSSION

Four main study projects were conducted to address scientific questions and establish feasibility (Figure 13).

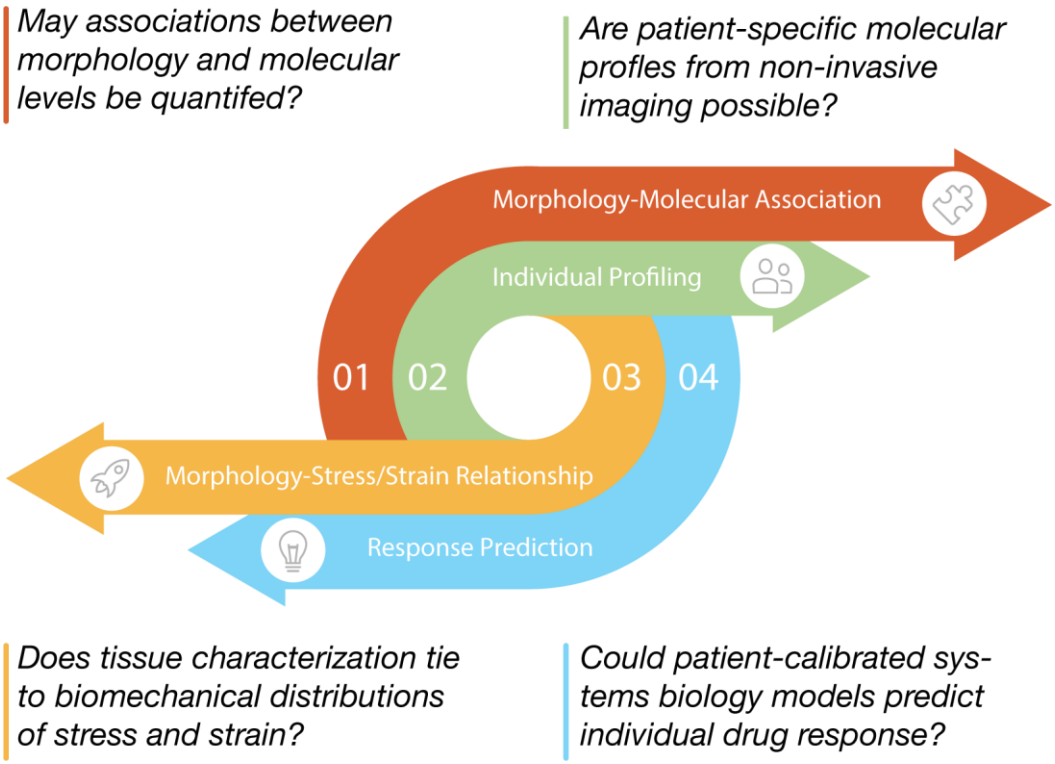


***Figure 13**: Four studies were undertaken to address critical scientific questions and establish feasibility.*

Additionally, during the course of study, several supporting projects applied portions of these methods to a growing number of clinical applications.

4.1 MORPHOLOGY-MOLECULAR ASSOCIATION

May associations between morphology and molecular levels be quantified?

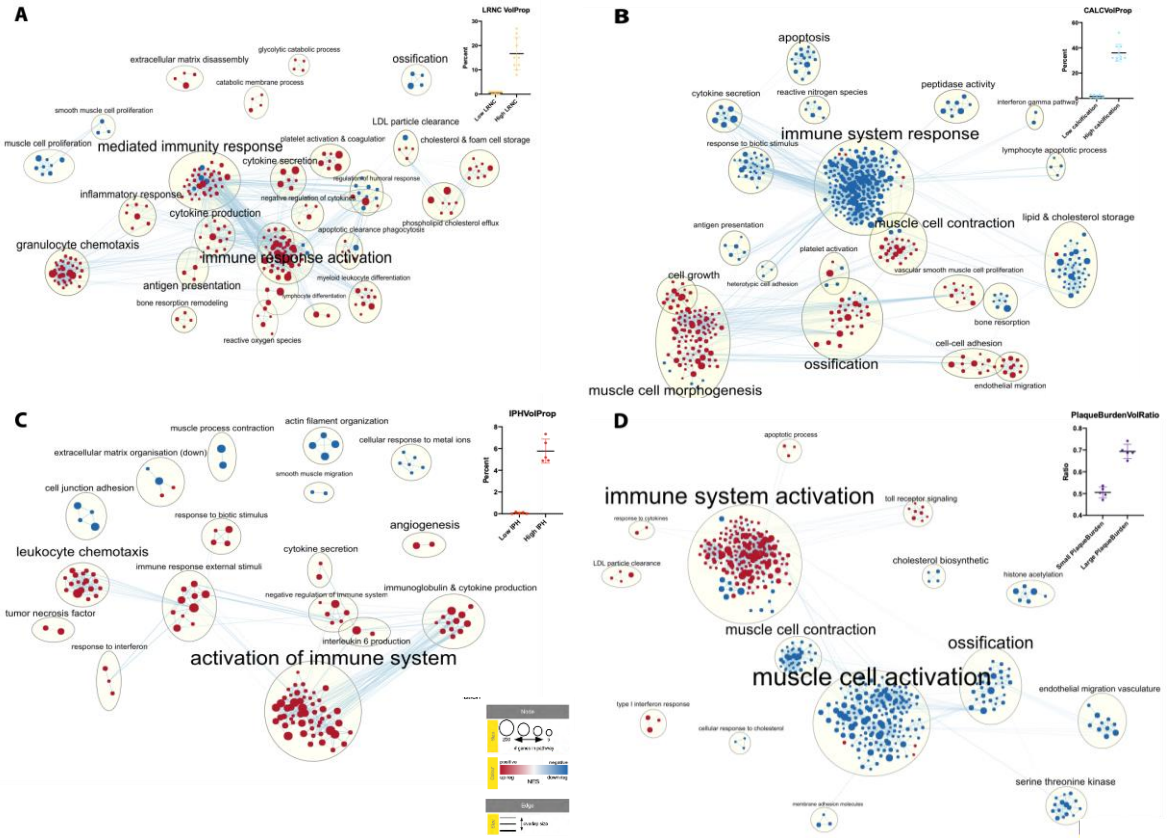


Figure 14: The ability to tie macroscopic plaque morphology to microscopic tissue types and further to molecular expression is demonstrated in Study 1.

Study 1 demonstrated that image analysis of conventional CTA for evaluating plaque morphology also reflects prevalent biological processes relevant to plaque instability (Figure 14). The study strengthens the concept of more sophisticated plaque phenotyping in risk stratification and managing patients with carotid stenosis and warrants further investigations in prospective clinical trials.

4.2 INDIVIDUAL PROFILING

Are patient-specific molecular profiles from non-invasive imaging possible?

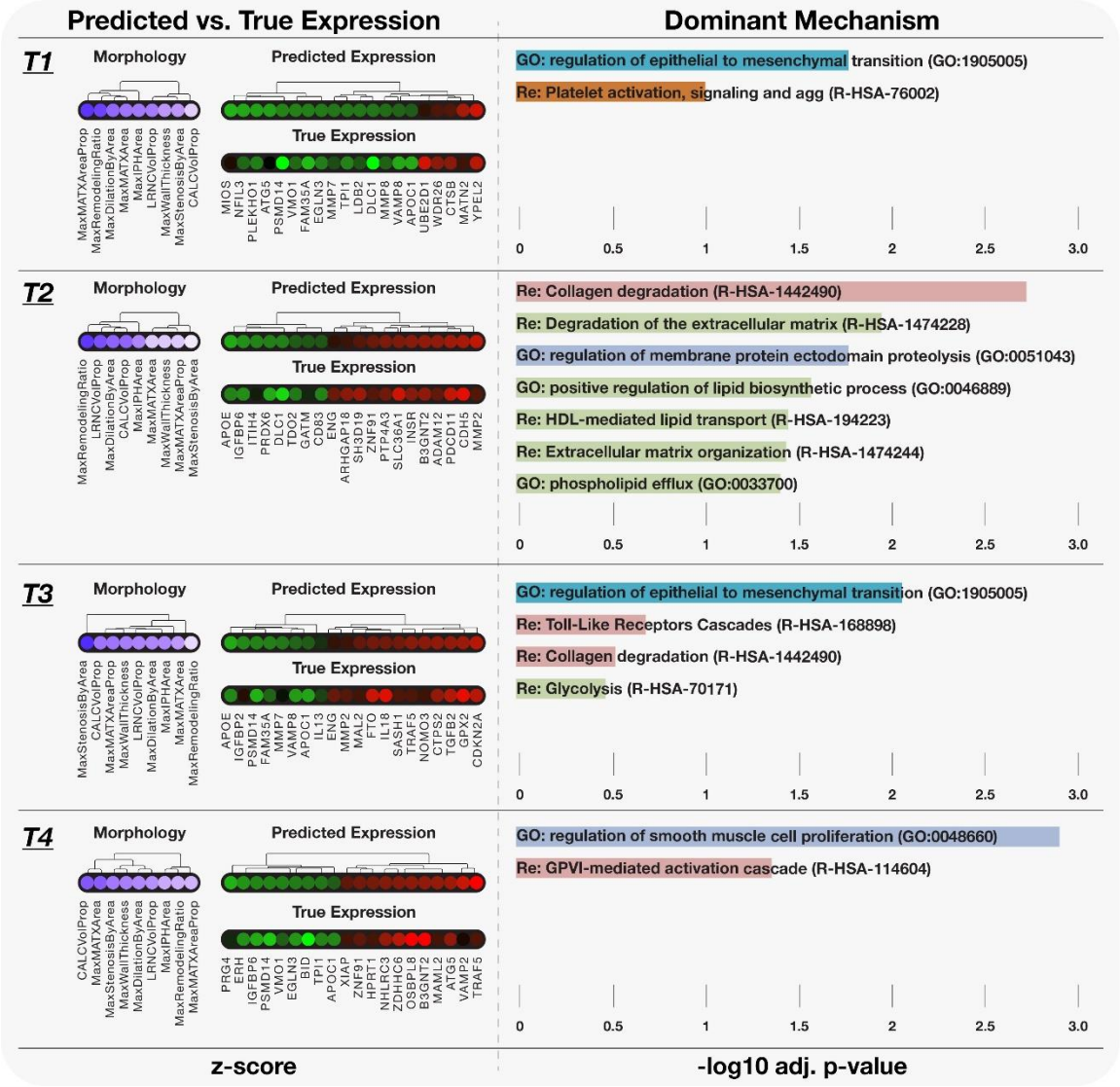


Figure 15: The ability to not only see cohort-level associations but to make reliable individual patient assessments/predictions of molecular quantities is established by Study 2. Study 1 identified a large set of statistically significant associations across many pathways but stopped short of determining this at the individual patient level. Study 2 extended this.

Study 2 demonstrated that statistically significant prediction of plaque biology using non-invasive CTA could be accomplished for individual patients (Figure 15). The results from this pilot study will serve as a basis for further exploration of non-invasive imaging to elucidate molecular signatures in atherosclerosis. Our results hold promise for individualized therapy in preventing myocardial infarction and ischemic stroke by categorizing patients according to the likely response of different therapeutic choices.

4.3 MORPHOLOGY-STRESS/STRAIN RELATIONSHIP

Does tissue characterization tie to biomechanical distributions of stress and strain?

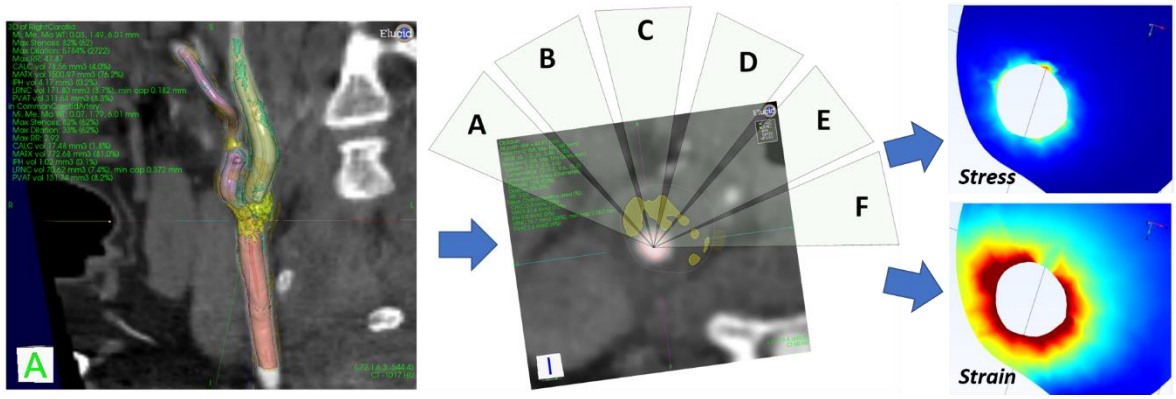


Figure 16: The question of whether biomechanical analyses provide new information incremental to plaque morphological assessment and whether it would suffice in the absence of accurate plaque morphology is taken up in Study 3. The findings suggest that biomechanical assessment is possible based on plaque morphology, but the converse is not. As a result, it may be concluded that plaque morphology assessment provides a broader base of information to support personalized medicine.

Study 3 demonstrated that histologically validated, patient-specific characterization of atherosclerotic plaque tissue components could be used to determine distributions of biomechanical stress and strain in a clinically relevant range for risk prediction (Figure 16). Moreover, these results demonstrate that biomechanical analyses, when based on patient-specific tissue characterization, provide biologically plausible distributions of stress and strain, but biomechanical analyses without such parameters did not.

4.4 RESPONSE PREDICTION

Could patient-calibrated systems biology models predict individual drug response?

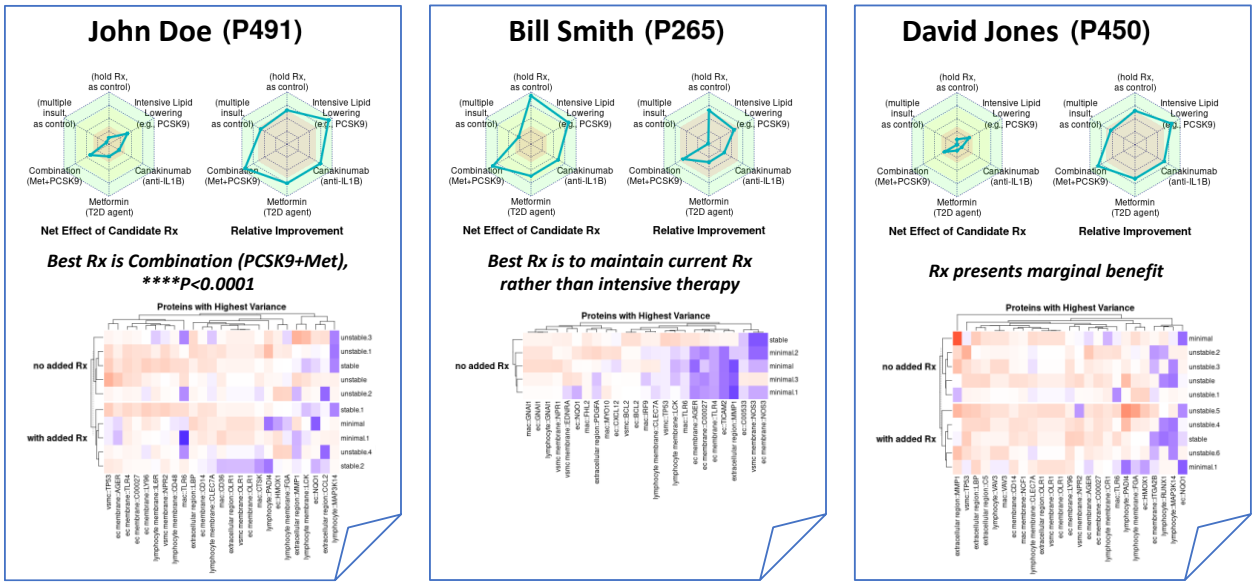


Figure 17. *Individualized patient treatment recommendations for three example patients, as might be utilized as a clinical decision support system incorporating the technique from this study. Recommendations generated by the software include the individual's absolute and relative radar plots, a statement on the benefit available through pharmacotherapy, and one or two heatmaps representing treated and baseline protein signatures. Patient "John Doe" exemplifies a patient with a highly unstable initial condition that may be improved with high confidence by pharmacotherapy. Patient "Bill Smith" represents a patient starting from a more stable initial condition where pharmacotherapy beyond conventional, preventive CVD medication would not be recommended. Patient "David Jones" represents a patient that would only marginally improve from pharmacotherapy and, based on the highly unstable starting point, should undergo procedural intervention.*

Study 4 developed a more comprehensive systems biology model of atherosclerotic plaque instability than has yet been reported, coupled with a practical response simulation method (Figure 17). Our approach effectively described and predicted changes in plaque stability following individual patient treatments, differentiating outcome as a function of the drug mechanism of action. Whereas there are an increasing number of treatment options for patients with CVD, our approach can provide clinical value for individualized therapies where patients could be selected for safe and relatively expensive therapies for more effective prevention of MI and IS.

5 CONCLUSIONS

We sought to address four fundamental questions that, taken together, could establish the feasibility of a non-invasive approach to improve diagnostic specificity for CVD at the individual level. From the included projects, we made the following conclusions:

- We determined that associations between macroscopic morphology may be made.
- We established the feasibility of estimating patient-specific molecular profiles from non-invasive radiological imaging.
- We found that tissue characterization provides the basis for clinically relevant assessment of biomechanical stress and strain, in addition to its use in molecular profiling.
- We developed the most comprehensive systems biology model of atherosclerosis than has yet been reported. We also demonstrated a method to calibrate it for individual patients to simulate likely responses to a range of drug classes.

Additionally, during the course of study, several supporting projects applied portions of these methods to a growing number of clinical applications in event prediction, drug response assessment, determination of flow limitations that generate angina, as well as statistical methodology such as using histological truth annotations for determination of test-retest performance.

In summary, results from this thesis will serve as a basis for practical uses of non-invasive imaging to categorize patients according to the likely response of therapeutic choices. This method can play a role in translating biological understanding through to improved clinical guidelines for optimizing care.

6 POINTS OF PERSPECTIVE

Given these results, a concrete roadmap of development is in view. Extrapolating the approach taken to transcriptomics for proteomics; extending the systems biology model to incorporate translation; and working with successively larger cohorts for model development and validation set directions for future work that we are now planning based on the feasibility established here.

Specifically, analysis outputs at various levels support clinical decision-making by informing the clinician of the current phenotype and the likely effect of different possible therapies. It can also provide tools to help discuss these options with the patient. The analyses offer recommendations based on the statistical significance of the likely improvement. They can compare potential recommendations to identify which of those considered exceeds others in the degree of improvement provided.

Such recommendations can determine a clinical action or inform decision-making that leads to a clinical action by allowing therapy to be tailored to the individual rather than based only on population statistics. Presently clinical guidelines have not been able to use such diagnostic specificity because there has been no means to do so. Individuals have different genetic predisposition, environmental exposures, and differing lifestyle habits. Both modifiable and non-modifiable risk factors influence what is best for that patient. The *in silico* systems biology model describes the disease, and this work provides a way to process and calibrate it for individual patients non-invasively. This enables the expected effect of therapies to be evaluated more precisely than previously possible. The benefit is that rather than referring to the population as a whole or, at best, sub-populations, actual molecular level effects may be considered for the specific patient.

The benefit of this approach has been widely understood in cancer treatment and is increasingly the norm. However, whereas cancer is generally informed by molecular diagnostics run on biopsied tumor tissue, it is impossible to biopsy atherosclerotic plaque tissues because it can cause the disruption we seek to avoid. As a result, a computer-based system that utilizes advanced techniques, including forms of artificial intelligence, can extend what the clinicians would otherwise be able to do by themselves. The tissues' features are generally too complex to be readily appreciated by a human observer without aid; the analyses described herein address that problem by evaluating the data at a far more granular level. To make such a decision support system practical is a mix of mathematical formulations, knowledge representation, and architecture in terms of user interfaces, reporting systems, and the backbone of computation. However, an engineering-only approach is insufficient; deep biological insights are mandatory to build such systems, which is the initial motivation for the multi-disciplinary approach.

The utility of any diagnostic system must address what can be done with the information. Presently numerous powerful therapies exist, both procedural, pharmaceutical, or combinations such as drug-eluting stents. By evaluating the individualized response to these therapies,

diagnostics become actionable by identifying the degree of improvement and the statistical significance of the computation.

Identification of likely responses at an individual patient level: More specifically, *in silico* systems biology model(s) may be generated, trained, and updated with patient-specific information, to create the baseline condition. The *in silico* systems biology model representing the baseline condition is then further updated to simulate one or more potential therapies based on the mechanism of action for each treatment to arrive at simulated conditions. Based on the results, therapeutic recommendations are computed with the absolute pathology and relative improvement, quantified, and expressed in numeric and report forms.

Quantifying actual responses at an individual patient level: After the patient has been on the recommended treatment regimen for sufficient time to elicit a therapeutic response, the simulated vs. new actual may be compared. Tangible improvement in the pathology indicates that the patient improved under the treatment. Further, suppose the actual measured changes to the protein levels were approximately as simulated. In that case, one can further determine that the treatment caused the improvement, and the method can be considered a surrogate endpoint for the treatment effect. The simulations must only be approximately correct to provide the intended utility in clinical practice.

Quantification of actual responses at a cohort level: Simulated and actual responses may also be assessed at a cohort level of patients or test subjects. This can be performed in the context of an observational, randomized, or other clinical trial design.

Detecting contraindications at an individual patient level: This approach may also be sued to simulate or screen for likely contra-indications. Deleterious side effects in the simulated condition are determined by evaluating how molecules are perturbed in the model, not just for evidence of improvement but also for side effects. That is, even if there is an apparent improvement in the pathology, there may be inadvertent other effects that are worse for the patient than the intended improvement, such as adverse reactions, toxicity, or likely future negative reactions. Likewise, assessment of actual response can be used for modifying treatments or conducting dynamic, combination, multi-stage, or adaptive clinical trial designs or individual patient management.

Screening tools for clinical trial enrichment to "select in" cases that increase the statistical power, to "select out" patients that decrease statistical power or which may suffer adverse events during the clinical trial period not related to the therapy: If the likely improvement of the patient is above an inclusion criteria threshold, one could select the patient for the clinical trial provided there are no other exclusion or inclusion criteria issues. Suppose there is a simulated adverse reaction of the patient or detected likely adverse event from other causes above an exclusion criteria threshold. In that case, one could recommend not enrolling the patient in the clinical trial.

7 ACKNOWLEDGEMENTS

There are many to acknowledge, as no work of substance occurs by one person; in this case, it is particularly so. I began this journey as a multi-disciplinary study in an explicit attempt to reach across traditional boundaries. I have been most fortunate to find willing partners in this endeavor, for which I am grateful.

I begin with **Ulf Hedin**, the most singularly responsible for allowing me this opportunity. I believe I surprised him quite a bit in 2017 when I asked whether he would accept me as a student. Whereas I am by no means the oldest person to undertake a Ph.D. program, I am far from the youngest. Ulf's initial inquiry into the matters we have taken up, and his foresight in establishing the tissue bank, were of central importance to a journey I started in 2008. I had left large-company industry to form an effort to innovate approaches to increase diagnostic power to what I then called "debilitating disease," of which cardiovascular disease is the largest. I did get a start, working methodically through histology first, but could not achieve the molecular level without paired specimens. Ulf had them, but more than that, he had curiosity, a rare mix of biological and clinical knowledge, and a multi-discipline laboratory rich in these capabilities to support him, all of which I needed and none of which I had. I recall him doing a double-take but soon said that we could try. Today we can all judge together whether his decision was a fruitful one. In any case, I am deeply indebted to him not only for what we have achieved but also for how we have achieved it. One only has one life to lead; Ulf has enriched mine with this activity beyond words.

Two others agreed to institutional roles without which this work would not have proceeded. **Ljubica Matic** and **Christian Gasser** would ultimately provide the solid base on which the work presented here could proceed. Each has provided knowledge and partnership for which I am very grateful.

At this point in these acknowledgments, I would like to highlight some of the many that created a personal and friendly environment that allowed a rather old and different type of person like myself to feel welcome and at ease. **Eva Karlöf** was the first to help me integrate. I was encouraged by her early on, and I have learned much from her. **Bianca Suur** and **Ula (Urszula) Rykaczewska** have a memorable spot. I recall one early trip being lost on the street, unsure where the lab was. Somehow they were there, greeted me with smiles, directed me where to go, and have retained those smiles through the years. **Mareia Talvitie** was with me in two early courses, and we had some fun with them. I will never forget the Finnish presentation at the end of one class, a real "hoot" in the context of the moment! **Katarina Wadén** and **Glykeria Karadimou** were consistently inviting, and **Hong Jin** is a bright face and presence that (my wife) Betty and I will not wish to part with.

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The last word goes to my wife, **Betty**. How can it be that 43 years after our first date, 38 of those being married, you still have time for me? We continue to have fun with the special time as we have gotten to know Stockholm almost as a second home and enjoying the opportunity to be students again. No written acknowledgment could suffice to express this.

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