Plasma Proteomic Profiling in Hypertrophic Cardiomyopathy Patients before and after Surgical Myectomy Reveals Post-procedural Reduction in Systemic Inflammation

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Abstract: Left Ventricular Outflow Tract (LVOT) obstruction occurs in approximately 70% of Hypertrophic Cardiomyopathy (HCM) patients and currently requires imaging or invasive testing for diagnosis, sometimes in conjunction with provocative physiological or pharmaceutical stimuli. To identify potential biomarkers of LVOT obstruction, we performed proteomics profiling of 1305 plasma proteins in 12 HCM patients with documented LVOT obstruction referred for surgical myectomy. Plasma was collected at the surgical preoperative visit approximately one month prior to surgery and then at the post surgical visit approximately 3 months later. Proteomic profiles were generated using the aptamer-based SOMAscan assay. Principal Component Analysis using the highest statistically significant proteins separated all preoperative samples from all postoperative samples. Further analysis revealed a set of 25 proteins that distinguished the preoperative and postoperative states with a paired t-test p value of <0.01. Ingenuity Pathway analysis facilitated the generation of protein interaction networks and the elucidation of key upstream regulators of the differentially expressed proteins such as interferon-γ, TGF-β1 and TNF. Biological pathways affected by the surgery included organ inflammation, migration and motility of leukocytes, fibrosis, vasculogenesis, angiogenesis, acute coronary events, endothelial proliferation, eicosanoid metabolism, calcium flux, apoptosis and morphology of the cardiovascular system. Our results indicate that surgical relief of dynamic outflow tract obstruction in HCM patients is associated with unique alterations in plasma proteomic profiles that likely reflect improvement in organ inflammation and physiological function.

Keywords: Hypertrophic Cardiomyopathy; proteomics; aptamer; cardiovascular disease; myectomy surgery

1. Introduction

Hypertrophic cardiomyopathy (HCM) is an autosomal dominant inherited disorder characterized by ventricular hypertrophy, often asymmetric in nature, frequently complicated by diastolic heart failure, left ventricular outflow tract (LVOT) obstruction, ventricular tachyarrhythmias, sudden cardiac death, microvascular angina and atrial fibrillation (reviewed in (1)). In HCM patients, the presence of LVOT obstruction can be a life threatening complication, independently associated with adverse outcomes, affecting approximately 70% (reviewed in (1)).
half of these affected patients, the outflow tract obstruction is dynamic, not apparent at rest but readily provicable with exercise. Thus, determination of clinically significant obstruction often requires physiologic testing in addition to imaging, which may not be readily available in some settings. Identification of a plasma biomarker associated with obstruction may help identify and risk stratify patients with LVOT obstruction and may also be used to measure efficacy of ablative therapies such as myectomy or alcohol septal ablation.

Aptamer based proteomic screening utilizes unique modified, single-stranded oligonucleotides that bind specifically and with high affinity to native target proteins and has been used to identify serum biomarkers in Duchenne Muscular Dystrophy [1], to assess serum biomarkers after myocardial injury [2] and identify potential biomarkers for HCM [3]. The method is commercially available and requires only fifty microliters of plasma or serum to measure the presence of 1305 proteins across 10 orders of magnitude. Studies using other methods have identified elevated levels of circulating cytokines in the plasma of HCM patients [4] and have suggested that measurements of brain natriuretic peptide may be useful in monitoring outcome after percutaneous alcohol septal ablation. Here we report the use of a commercially available aptamer based proteomics platform, SOMAscan (SomaLogic, Boulder, CO), to identify biomarkers associated with LVOT obstruction in patients with HCM by measuring plasma levels before and after surgical myectomy. In this study, we demonstrate that plasma proteomic profiles can distinguish the preoperative from the postoperative state through changes in proteins linked to pathways that regulate inflammation, leukocyte migration, fibrosis, angiogenesis and vasculogenesis, potentially implicating these processes as important in the pathogenesis of LVOT obstruction in HCM and identifying potential new therapeutic targets.

2. Results

2.1. Patient Cohort Characteristics

The 12 patients chosen randomly from HCM patients referred for surgical myectomy are characterized in Table 1. The patients varied in age from 37 to 76. Nine of twelve were female and eleven of 12 had NYHA heart failure classification of 3 or greater. Two of the patients carried pathogenic Mybpc3 mutations, 5 patients had no pathogenic mutations found during screening and 5 did not have a record of genetic screening. Two out of twelve patients had a history of atrial fibrillation and two of twelve had a history of ventricular tachycardia or ventricular fibrillation leading to ICD placement. Eleven of twelve had medical comorbidities in addition to HCM. Eleven out of twelve were taking beta blockers. LVOT gradients were documented for all patients, either at rest or with provocation, ranging from 30 to 150 mm Hg. Eight of twelve had at least mild mitral regurgitation. All 12 patients underwent surgical myectomy, while two had concurrent mitral valve surgery, two had concurrent coronary artery bypass grafting and two had aortic valve replacement for concurrent aortic stenosis. The two patients with atrial fibrillation had concurrent MAZE procedures. All had no residual LVOT gradient on follow up echocardiogram done around the time

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of the postoperative visit.

**Table 1- Patient demographics and clinical characteristics for all cohorts**

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---------|---|---|---|---|---|---|---|---|---|----|----|----|
| Sex     |   |   |   |   |   |   |   |   |   |    |    |    |
| Age     | 37 | 43 | 51 | 56 | 75 | 71 | 78 | 60 | 52 | 29 | 63 | 77 |
| BMI     | 30 | 39 | 35 | 32 | 25 | 27 | 28 | 30 | 29 | 33 | 34 | 36 |
| HTN     | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |
| DM2     | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |
| OSA     | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |
| COPD    | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |
| CAD     | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |
| HLD     | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |
| Spinal Stenosis | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  | y  |

**2.2. SOMAscan plasma proteomics demonstrates within person stability of distinct protein fingerprints**

SOMAscan analysis was performed on paired plasma samples from 12 patients. We wanted to first understand in more detail the proteome profiles of these samples and the relationships of the individual pre- and post-surgery samples based on relative expression of all 1,305 proteins. Consequently, we performed hierarchical clustering using all samples across all proteins (Fig. 1). Hierarchical clustering sorts samples by similarity of protein expression patterns. Samples with a more comparable expression pattern cluster together and separate from samples with a more dissimilar expression pattern. This hierarchical cluster analysis of all samples with all proteins demonstrated that each paired Pre/Post patient sample clustered together and separated from all other patients (Fig. 1). This result indicates that the overall expression profile of all proteins is more closely related within a patient than between Pre- and Post-surgery, suggesting that each person has a unique overall plasma protein fingerprint distinct from any other person.

**Figure. 1.** Hierarchical clustering of serum proteomic profiles sorts by patient identity

**2.3. SOMAscan enables the detection of myectomy-related protein expression and identification of differentially expressed proteins in plasma that distinguish between the preoperative and postoperative state**

Protein expression levels were compared in the preoperative and postoperative states and sorted by median fold change. SOMAscan analysis revealed 79 out of 1305 proteins whose expression levels were significantly different (p < 0.05) in plasma from matched Post-surgery versus
Pre-surgery patients. 29 proteins were elevated Post-surgery in patients undergoing myectomy, while 50 proteins were decreased as compared to the Pre-surgery samples. The 79 upregulated and down regulated proteins with the greatest degree of differential expression are listed in Table 2, with associated gene symbols and paired t-test p-values. Adjusted p-values for multiple comparison testing using the Benjamini-Hochberg method are also shown. After adjustment, no individual marker reached statistical significance (p<0.05), most likely due to sample size limitations. We have observed this in numerous SOMAscan studies with small sample size. Nevertheless, we and others have been able to further validate various proteins with unadjusted p-values.
Table 2. Up-regulated and Down-regulated proteins between Hypertrophic Cardiomyopathy Patients before (PRE) and after Surgical Myectomy (POST)

| Increased in POST as compared to PRE | TargetFullName | Target | Gene Symbol | p-value | BH adj. p-value | Mean FC | Median FC |
|-------------------------------------|---------------|--------|-------------|---------|----------------|---------|----------|
| SL000522 Macrophage metalloelastase | MMP9-12 | MMP2 | 4.376E-05 | 0.0525076 | 1.31 | 1.68 |
| SL000774 Cytochrome b5 reductase | CYP2D6 | CYP2D6 | 2.07E-05 | 0.0203103 | 1.79 | 2.64 |
| SL000684 Periostin | Periostin | POSTN | 8.727E-05 | 0.0562200 | 1.34 | 1.36 |
| SL000946 IgG lambda light chain | IGGL | IGL | 7.02E-05 | 0.0290191 | 1.23 | 1.23 |
| SL001167 E-cadherin-like 13 | CLN13 | CLN13 | 1.27E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001741 Collectin-12 | COLC12 | COLC12 | 1.32E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001719 Carbonic anhydrase 2 | CA2 | CA2 | 1.33E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001959 40S ribosomal protein S3 | 40S | 40S | 1.36E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001206 Prealbumin | PREALB | PREALB | 1.37E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001729 Transthyretin | transthyretin | TTR | 1.38E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001850 Leucine-rich repeat transmembrane protein 4 | LRR2 | LRR2 | 1.40E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001452 Interleukin-1 beta | IL-1B | IL-1B | 1.41E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001399 Rho guanyl nucleotide exchange factor | RHOG | RHOG | 1.42E-05 | 0.0070069 | 1.07 | 1.07 |
| SL001993 Bone morphogenetic protein 6 | BMP6 | BMP6 | 1.43E-05 | 0.0070069 | 1.07 | 1.07 |
| SL002089 Low affinity immunoglobulin gamma Fc receptor IIb | FCGRIIb | FCGRIIb | 1.44E-05 | 0.0070069 | 1.07 | 1.07 |
| SL002058 Interleukin-18 binding protein | IL-18BP | IL-18BP | 1.45E-05 | 0.0070069 | 1.07 | 1.07 |
| SL002041 Serpin A1 | SERPINA1 | SERPINA1 | 1.46E-05 | 0.0070069 | 1.07 | 1.07 |
| SL002160 Leukocyte elastase 1 | LEPA | LEPA | 1.47E-05 | 0.0070069 | 1.07 | 1.07 |
| SL002206 Complement component C3 | C3 | C3 | 1.48E-05 | 0.0070069 | 1.07 | 1.07 |

Decreased in POST as compared to PRE

| Decreased in POST as compared to PRE | TargetFullName | Target | Gene Symbol | p-value | BH adj. p-value | Mean FC | Median FC |
|--------------------------------------|---------------|--------|-------------|---------|----------------|---------|----------|
| SL000436 Hepcidin | HAMP | HAMP | 1.38E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000403 Fetal tourus homeodomain-like protein 1 | FTLM | FTLM | 1.39E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000420 Phosphorylase mucosa 1 | PLOM1 | PLOM1 | 1.40E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000709 Cystatin-F | CSTF | CSTF | 1.41E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000730 Leukocyte Adhesion 4-4-hydroxide | LEAH4 | LEAH4 | 1.42E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000333 Vascular endothelial growth factor-A, isoform 12F | VEGFA | VEGFA | 1.43E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000835 Angiopoietin receptor protein 1 | AGFR1 | AGFR1 | 1.44E-05 | 0.0070069 | 1.36 | 1.36 |
| SL000901 Complement component C3 | C3 | C3 | 1.45E-05 | 0.0070069 | 1.36 | 1.36 |
| SL001052 Complement component C4 | C4 | C4 | 1.46E-05 | 0.0070069 | 1.36 | 1.36 |

Figure 2A shows a heatmap of the top 25 proteins listed in Table 1 with the most significant (p<0.01) differential expression between the matched pairs of Post- and Pre-surgery patients that distinguish the preoperative and postoperative states in obstructive HCM and highlights the relative minimum and maximum concentrations for each protein in each patient. While the baseline
Pre-surgery levels for each protein are different for each patient, the relative changes of these proteins (increase or decrease in Post versus Pre) trend in the same direction for most or all patients for this set of proteins as visualized by the change in color (Fig. 2A). Individual markers within this group either decreased or increased in a consistent manner across all patients, suggesting that subsets of differentially expressed genes may associate with either the preoperative or postoperative state. For example, POSTN is increased at Post, while LTA4H is decreased at Post. In Figure 2B, Box Whisker plots of the Pre and Post samples illustrate the difference in SOMAscan expression levels for six representative targets linked to myectomy: POSTN, MMP12, CDON, NAMPT, HAMP, and LTA4H are included. Several of the proteins impacted by myectomy (POSTN, MMP12, CDON, NAMPT, HAMP, LTA4H) have been previously reported to be altered in cardiovascular disease, cardiomyopathy or vascular disease with effects mediated through inflammatory mechanisms [5-13].

Figure 2. A. Heat Map of 25 proteins differentially expressed (p < 0.01) in the preoperative and postoperative states, shown by patient. In the colormap, red denotes upregulation and green denotes downregulation. B. Box Whisker plots of selected top proteins.

While we did not anticipate that an individual biomarker can distinguish the preoperative state from the postoperative state in HCM patients undergoing surgical myectomy with high accuracy, it has become apparent that biomarker panels incorporating multiple proteins improve accuracy. To assess whether a set of the statistically most significant differentially expressed proteins is able to accurately discriminate between Post and Pre, we performed principal component analysis (PCA) using the log2 transformed expression levels of the top 11 differentially expressed proteins (p<0.003), PCA reveals excellent separation of the pre-operative and post-operative states (Fig. 3) in two dimensions (Fig. 3). The first principal component accounts for 25.53% of the variance and the second principal component for 22.32% of the variance. This analysis demonstrates that the SOMAscan-derived proteomics data contain a significant component that differentiates between pre-operative and post-operative states.
Figure 3. Principal Component Analysis of 11 differentially expressed plasma proteins from HCM patients reveals excellent separation between preoperative and postoperative samples. Blue circles, Post; Green circles, Pre.

2.4 Ingenuity Pathway Analysis reveals protein interaction networks, upstream regulators and biological processes relevant to HCM

We then performed Ingenuity Pathway Analysis using the 79 myectomy-associated proteins to analyze the SOMAscan results in the context of the signaling pathways they are known to participate in. Network analysis of differentially expressed proteins revealed 3 statistically highly significant, distinct interaction networks (Fig. 4). The first, most extensive network includes the growth factors EGF and VEGF as important nodes that are reduced in the postoperative state and linked to reduction of pro-inflammatory cytokines and signaling molecules related to inflammation, including IL25, TNSF12, TNFSF14, TNFRSF12A, TNFRSF18 and IL10RB (Fig. 4A). Interestingly, many intracellular signaling molecules are present as network nodes but not present in the actual proteomic dataset, such as ERK1, SRC, RAS, tyrosine kinase. Lack of signal for these nodes is not surprising since the proteomic dataset is from proteins circulating in plasma and they are either not detected or not represented in the SOMAscan assay. Their presence in the network suggests important links between circulating extracellular proteins and intracellular signaling pathways. A second network includes MMP12 as a central node with increased expression in the postoperative state accompanied by increased expression of additional extracellular matrix (ECM) proteases, ADAMTS15 and elastase, as well as the ECM protein peristin (POSTN) and the intercalated disc protein, CDON (Fig. 4B). This network is linked to intracellular AKT signaling and various ECM proteins. A third network is focused on VEGFA, Ap1, LDL, GSTP1 and is linked to intracellular NOS and NFκB signaling (Fig. 4C).
Network of VEGF, NOS and NF-κB signaling. Red indicates upregulation and green denotes downregulation in Post. Proteins are coded by shape; square: cytokine, vertical rhombus: enzyme, horizontal rhombus: peptidase, trapezoid: transporter, ellipse: transmembrane receptor, circle: other.

Modeling the links between myectomy-associated proteins and shared upstream regulatory proteins was particularly informative. In Figure 5A, the upstream regulators that are most significantly enriched by the input of the 79 proteins are shown. Analysis of the predicted positive upstream regulators for the differentially expressed proteins converged on Tumor Necrosis Factor (TNF), Interferon γ (IFNγ) and Transforming Growth Factor β1 (TGFβ1) as most significant. 30 out of the 79 proteins are predicted downstream targets of the pro-inflammatory cytokine TNF (Fig. 5B), while 24 proteins are downstream of IFNγ (Fig. 5C) and 28 proteins are downstream of TGFβ1 (Fig. 5D). Several proteins mentioned above as being linked to cardiovascular disease, cardiomyopathy, or vascular disease are highlighted by the red arrows in the upstream regulator networks. The proteins that are increased after surgery are denoted by red symbols and the ones that are decreased by green symbols. These results indicate that these signaling nodes are likely involved in the dysregulation of a sizeable portion of the top 79 proteins in the myectomy signature. Predicted negative upstream regulators of interest include Epidermal Growth Factor (EGF), FOS and CD28, shown with their downstream targets (Fig. 6A-C). An additional predicted positive upstream regulator, CSF1, is also shown with its downstream targets (Fig. 6D)

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**Figure 5.** Upstream Regulator Analysis shows significant effects of surgical myectomy on proteins regulated by TNF, IFNγ and TGFβ1. Upstream regulators that best explain the observed expression changes in the input 79 protein list as their targets. A. Analysis of upstream regulators ranked by -log p value. B. Downstream targets of TNF. C. Downstream targets of IFNγ. D. Downstream targets of TGFβ1. Red indicates upregulation and green denotes downregulation in Post. Proteins are coded by shape; square: cytokine, vertical rhombus: enzyme, horizontal rhombus: peptidase, trapezoid: transporter, ellipse: transmembrane receptor, circle: other. Links are color-coded as red: leads to activation, blue: leads to inhibition, yellow: findings inconsistent with state of downstream protein, black: effect not predicted. Red arrows indicate proteins of particular interest and relevance.

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**Figure 6.** Upstream Regulator Analysis shows significant effects of surgical myectomy on proteins regulated by A. EGF, B. FOS, C. CSF1 and D. CD28. Red indicates upregulation and green denotes downregulation in
Post. Proteins are coded by shape; square: cytokine, vertical rhombus: enzyme, horizontal rhombus: peptidase, trapezoid: transporter, ellipse: transmembrane receptor, circle: other. Links are color-coded as red: leads to activation, blue: leads to inhibition, yellow: findings inconsistent with state of downstream protein, black: effect not predicted. Red arrows indicate proteins of particular interest and relevance.

Among the significantly affected functional categories, enrichment for biological functions linked to “Inflammation of Organ” were most prominent, followed by biological processes associated with leukocyte migration, cell movement of leukocytes, fibrosis, vasculogenesis, angiogenesis, development of vasculature, chronic inflammatory disorder, and cell movement of mononuclear leukocytes (Fig. 7). Other highly enriched key bio functions of particular interest with regard to cardiomyopathy include acute coronary event, myocardial infarction, acute myocardial infarction, and abnormal morphology of cardiovascular system, and morphology of cardiovascular system (Fig. 7). Metabolism of eicosanoid and synthesis of eicosanoid further support the notion of inflammatory processes contributing to the surgery effect (Fig. 7). Figure 8 highlights in detail the 23 proteins linked to vascular functions among the 79 proteins (Fig. 8A), the 19 proteins associated with fibrosis (Fig. 8B), the 11 proteins linked to cardiovascular infarction (Fig. 8C), and the 14 proteins linked to eicosanoid metabolism, synthesis, and release (Fig. 8D).

Figure 7. Biological Functions affected by surgical myectomy. Biological functions that are significantly enriched by the 79 input protein list ranked by -log p value.

Figure 8. Biological Functions linked to altered plasma protein profiles after surgical myectomy with predicted protein interactions. A. Biological network linked to vascular function. B. Biological network linked to fibrosis. C. Biological network linked to myocardial injury. D. Biological network linked to eicosanoid metabolism. Red indicates upregulation and green denotes downregulation in Post. Proteins are coded by shape; square: cytokine, vertical rhombus: enzyme, horizontal rhombus: peptidase, trapezoid: transporter, ellipse: transmembrane receptor, circle: other. Links are color-coded as red: leads to activation, blue: leads to inhibition, yellow: findings inconsistent with state of downstream protein, black: effect not predicted. Red arrows indicate proteins of particular interest and relevance.
3. Discussion

We have found that plasma protein profiles from HCM patients with LVOT obstruction can distinguish the preoperative from the postoperative state, and surgical myectomy results in a reduction of circulating plasma proteins associated with a proinflammatory state. The association between HCM and a proinflammatory state is consistent with previous reports [4, 14], but our study is the first, to the best of our knowledge, to demonstrate a potential improvement after surgical myectomy. The potential mechanisms by which HCM leads to systemic inflammation are not clear, but are associated with myocardial fibrosis, which may be secondary to cardiomyocyte injury. A possible mechanism by which surgery to relieve outflow tract obstruction alleviates myocardial injury may involve reduction of subendocardial ischemia from elevated LV filling pressures. In this context it is interesting to see the reduction in nicotinamide phosphoribosyltransferase (NAMPT) after surgery. This enzyme plays a significant role in cerebral ischemia [15], hypertension, atherosclerosis, heart failure [10] and ischemic heart disease [16] as well as inflammatory processes, and NAMPT inhibitors have been shown to protect against neuronal injury in animal models (reviewed in [11]).

Another interesting finding is that circulating matrix associated proteases such as MMP12 show increased levels, along with circulating matrix proteins, in the postoperative state. One possible explanation is that the increase in matrix remodeling enzymes is a consequence of wound healing and the postoperative state, although one would expect that surgical wound healing would be completed by the three month follow up visit. A more intriguing possibility is that surgical relief of LVOT obstruction is associated with prolonged extracellular matrix remodeling in the HCM heart that retards the development of interstitial fibrosis seen in advanced cases.

A potential role for angiogenesis in the pathogenesis of HCM has not previously been established. A role for angiogenesis in cardiac hypertrophy, however, has been demonstrated repeatedly in experimental models (reviewed in [17]). Increased capillary vascularity is thought to support the increased circulatory and metabolic demands of the hypertrophied cardiomyocyte. Increased angiogenesis in HCM may also occur in response to subendocardial ischemia. A reduction in circulating proangiogenic factors after myectomy, as suggest by the data in this study, is an unexpected finding, but again may reflect a reduction in subendocardial ischemia after reduction of elevated filling pressures.

Our work has also identified potential upstream regulators of systemic inflammation and fibrosis, such as TNFα, IFNγ and TGFβ1. EGF, another identified upstream regulator, is also known to promote angiogenesis through induction of autocrine VEGF expression [18], to regulate inflammation through its effects on TNSRsf12A (also known as FN14) [19] and to regulate matrix turnover through effects on MMP12, which itself regulates angiogenesis and inflammation [20]. The cellular oncogene c-fos, also found in our screen for upstream regulators, promotes angiogenesis through the induction of VEGF [21]. CSF1, another upstream regulator identified in our analysis, controls the production, differentiation and function of macrophages [22] and thus is also an important regulator of inflammation. CD28, another upstream regulator, is involved in T-cell activation, induction of cell proliferation and cytokine production and promotion of T-cell survival, and thus is also involved in regulation of the immune response [23]. These upstream regulators may provide potential therapeutic targets in obstructive HCM.

A recent study examined plasma proteomic profiles in patients with aortic stenosis before and after transcatheter valve replacement [24]. Aortic stenosis results in fixed outflow tract obstruction from abnormal narrowing of the aortic valve, and transcatheter aortic valve replacement relieves the obstruction. In HCM, LVOT obstruction is dynamic, and to date a comparison of plasma profiles in dynamic vs. fixed obstruction has not been done. The published aortic stenosis dataset using the same SOMAscan technology implicates MAP kinase signaling, HIPPO signaling and focal...
adhesion pathways as important mediators of myofibroblast activation. Our findings in HCM do not appear to involve these pathways and is consistent with different underlying pathophysiology for LVOT obstruction in HCM compared with the pathogenesis of aortic stenosis. The presence of an outflow tract gradient is thus not sufficient to account for the same proteomic changes seen in either condition. Nevertheless, 12 out of the 79 proteins identified in our study were also found as differentially expressed after surgery in the study by Aguado et al. [24]. This includes 3 proteins increased after surgery (MMP12, RPS3, CD5L) and 9 proteins decreased after surgery (HAMP, PLA2G1B, TNFSF12, MB, DCTN2, C3, FAM107B, ACP5, BCL2L2).

Our study is the first to measure plasma proteomics in patients with obstructive HCM before and after surgical myectomy and to demonstrate potential pathogenic pathways affecting inflammation, fibrosis and angiogenesis. Furthermore, our study identifies potential upstream regulatory therapeutic targets for LVOT obstruction in a human HCM population. Targeting of these putative upstream regulators may reduce inflammation, fibrosis and angiogenesis and thus may possibly be beneficial in the treatment of HCM, pending future validation studies.

4. Materials and Methods

4.1 Study Patients

A total of 12 patients with clinically documented HCM referred and scheduled for surgical myectomy were approached for written informed consent to participate in the study. Those who consented underwent a venous blood draw at their preoperative evaluation, within 4 weeks of their scheduled procedure. Follow up blood draws were performed at their HCM clinic postoperative visit, approximately 3 months after surgery. Sample collection was approved by the Tufts University/Medical Center Health Sciences Institutional Review Board under IRB protocol # 9487. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki. Patient characteristics were obtained from the medical record and are shown in Table 1.

4.2 Blood sample processing

Blood samples were collected in K$_2$EDTA tubes and centrifuged at 2000g for 15 minutes at 4°C to separate cells from plasma. The supernatant plasma was then aliquoted and stored at -80°C.

4.3 SOMAscan Proteomics Profiling

Pre- and Post-surgical EDTA plasma samples were analyzed using the commercially available, aptamer based SOMAscan manual assay (version 1.3k) for human plasma that measures 1305 proteins (SomaLogic, Boulder, CO), through the SomaLogic trained and certified assay site, BIDMC Genomics, Proteomics, Bioinformatics and Systems Biology Center at Beth Israel Deaconess Medical Center [https://www.bidmc.org/research/core-facilities/genomics-proteomics-core]. The method is highly multiplexed, sensitive, specific, quantitative, and reproducible across 10 orders of magnitude (femtomolar to micromolar concentrations) [25, 26], requiring only 50 µl of patient plasma. For each run, a no protein negative buffer control and five pooled plasma samples were run with the patient samples for normalization and calibration. Sample data was normalized to remove hybridization variation within a run followed by median normalization across all samples to remove other assay biases within the run and finally calibrated to remove assay differences between runs. All samples passed all the SomaLogic standard quality control and normalization criteria for the manual 1.3k assay. These include hybridization normalization, plate scaling, median normalization, and calibration.

4.4 Bioinformatics Analysis
Before application of the analytical methods to the proteomic data, SOMAscan relative fluorescence units (RFUs) were log transformed. Normalized data were initially analyzed by hierarchical clustering as previously described [27], using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). The paired t-test was applied to log2 transformed data and a p-value cutoff < 0.05 was considered significant. The Benjamini-Hochberg (BH) procedure was also employed to correct for testing multiple hypotheses. Since due to small sample size the BH correction did not reach a BH p-value <0.05, the paired t-test was used as the primary cutoff. The mean and median fold-change (FC) of protein expression was calculated for the significant proteins. Principal Component Analysis (PCA) was performed and illustrated using XLSTAT (Addinsoft, Long Island City, NY). Box-and-whisker plots were generated using XLSTAT.

To acquire new insights into potential pathophysiological pathways associated with LVOT obstruction in patients with HCM based on myectomy-specific serum protein signatures, pathway and functional analysis were performed using Ingenuity Pathway Analysis (IPA) software, a commercially available platform for analysis, integration and interpretation of data derived from omics experiments (Qiagen Bioinformatics, Redwood City, CA) [28]. Data analysis and interpretation is based on the use of proprietary algorithms in conjunction with a comprehensive, highly curated Ingenuity Knowledge Base that allows identification of key pathways, upstream regulators, biological processes, protein interaction networks, disease associations and small molecule effectors. Ingenuity Pathway Analysis (IPA) uses enrichment analysis-based approaches to calculate the significance of observing a candidate protein set within the context of biological systems. Core analysis, including canonical pathways, upstream regulators and network analysis, was performed.

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**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| LVOT         | Left Ventricular Outflow Tract |
| HCM          | Hypertrophic Cardiomyopathy |
| PCA          | Principal Component Analysis |
| ECM          | Extracellular Matrix |
| TNF          | Tumor Necrosis Factor |
| IFNγ         | Interferon γ |
| TGFβ1        | Transforming Growth Factor β1 |
| POSTN        | Periostin |
| NAMPT        | Nicotinamide Phosphoribosyl-transferase |
| EGF          | Epidermal Growth Factor |
| VEGF         | Vascular Endothelial Growth Factor |

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