Immune-Mediated Inflammation May Contribute to the Pathogenesis of Cardiovascular Disease in Mucopolysaccharidosis Type I

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Abstract

Background
Cardiovascular disease, a progressive manifestation of α-L-iduronidase deficiency or mucopolysaccharidosis type I, continues in patients both untreated and treated with hematopoietic stem cell transplantation or intravenous enzyme replacement. Few studies have examined the effects of α-L-iduronidase deficiency and subsequent glycosaminoglycan storage upon arterial gene expression to understand the pathogenesis of cardiovascular disease.

Methods
Gene expression in carotid artery, ascending, and descending aortas from four non-tolerized, non-enzyme treated 19 month-old mucopolysaccharidosis type I dogs was compared with expression in corresponding vascular segments from three normal, age-matched dogs. Data were analyzed using R and whole genome network correlation analysis, a bias-free method of categorizing expression level and significance into discrete modules. Genes were further categorized based on module-trait relationships. Expression of clusterin, a protein implicated in other etiologies of cardiovascular disease, was assessed in canine and murine mucopolysaccharidosis type I aortas via Western blot and in situ immunohistochemistry.

Results
Gene families with more than two-fold, significant increased expression involved lysosomal function, proteasome function, and immune regulation. Significantly downregulated genes were related to cellular adhesion, cytoskeletal elements, and calcium regulation. Clusterin...
gene overexpression (9-fold) and protein overexpression (1.3 to 1.62-fold) was confirmed and located specifically in arterial plaques of mucopolysaccharidosis-affected dogs and mice.

**Conclusions**

Overexpression of lysosomal and proteasomal-related genes are expected responses to cellular stress induced by lysosomal storage in mucopolysaccharidosis type I. Upregulation of immunity-related genes implicates the potential involvement of glycosaminoglycan-induced inflammation in the pathogenesis of mucopolysaccharidosis-related arterial disease, for which clusterin represents a potential biomarker.

**Introduction**

Mucopolysaccharidosis type I (MPS I), caused by a deficiency of the lysosomal enzyme α-L-iduronidase (IDUA), results in shortened lifespan, multisystemic somatic involvement, and variable neurocognitive degeneration because of accumulation of heparan sulfate (HS) and dermatan sulfate (DS) glycosaminoglycan (GAG) substrates in body tissues such as brain, soft tissues, chondrocytes, liver, and spleen [1]. Cardiovascular disease is a cardinal manifestation of MPS I, characterized by progressive thickening and compromised function of the heart valves, left ventricular hypertrophy, and diffuse coronary artery stenosis [2–6].

The advent of treatments to replace the missing IDUA enzyme, whether with intravenous enzyme replacement therapy (ERT) or via hematopoietic stem cell transplant (HSCT), has enabled MPS I patients to survive into adulthood [7, 8]. Although ERT and HSCT are able to mitigate many symptoms of MPS I, clinical experience has demonstrated that these treatments attenuate, but do not cure, the disease. Certain tissues remain resistant to treatment and continue to manifest GAG storage. Consequently, as MPS I patients survive into adulthood they face a different set of potentially life-threatening disease complications such as those involving the cardiovascular system [9, 10]. Cardiac sudden death, left-sided valvular insufficiency, ventricular dysfunction, and coronary intimal proliferation with stenosis all have been reported in stably treated patients [11–14]. Accumulation of GAG within cardiovascular structures in the face of ongoing treatment is the likely origin of these symptoms, as well as childhood-onset carotid intima-media thickening and abnormally reduced elasticity [15–19].

The pathogenesis and etiologies of treatment resistance of cardiovascular disease in MPS I are not well characterized, but studies in the murine and canine models of the disease indicate that accumulation of undegraded HS and DS GAG in the heart, valves, and vasculature alone do not adequately describe the pathophysiology. Both untreated and treated MPS I canines develop similar cardiovascular findings to human MPS I, with cardiac hypertrophy, nodular valve thickening, and vascular smooth muscle proliferation of the aorta with luminal stenosis [20–22]. Detailed histopathology of canine aortic lesions demonstrates vascular smooth muscle proliferation, activated CD68+ macrophages, and fragmented elastin fibrils in addition to GAG storage [23]. The murine MPS I model also manifests with cardiac enlargement, valvular thickening and dysfunction, and dilatation of the aorta with vascular wall thickening with elastin fibril degradation [24, 25]. Gene expression studies are a useful method to identify potential mechanisms of disease progression, but have not been comprehensively assessed for cardiovascular disease in any model of MPS I. The primary focus of expression studies for the mucopolysaccharidoses has been neurodegeneration in the Sanfilippo syndromes (MPS III) and Sly syndrome (MPS VII).
Assessment of aortic mRNA expression for dogs with MPS I and VII, and mice with MPS VII has centered on quantification of cytokine, complement, and other inflammation-related genes [31–34]. Herein, we report alterations in arterial gene and protein expression in the canine MPS I model system, the identification of a potential marker for MPS I cardiovascular disease, and data supporting the hypothesis that a GAG-induced inflammatory process is responsible for the pathogenesis of MPS I cardiovascular disease.

Materials and Methods

Test animals and husbandry

This study was reviewed and approved by the Institutional Animal Care and Use Committees of both Iowa State University (IACUC #12-04-5791-K) and the Los Angeles Biomedical Research Institute at Harbor-UCLA (LA BioMed IACUC #20013-01). Studies were conducted in compliance with both USDA and NIH guidelines for the use of dogs in research. Four MPS I (IDUA-/-) canines were produced from artificial insemination breedings of parental stock, diagnosed via α-L-iduronidase enzyme assay and PCR, and maintained at Iowa State University until 1 year of age, after which they were transported to LA BioMed, an AAALAC accredited facility overseen by a licensed veterinarian. Animals had ad libitum access to food (standard Teklad canine lab chow) and water, housing with enrichment, and a 12 hour light/dark cycle. Housing consisted of expanded mesh flooring, and involved housing with compatible conspecifics as required for social species unless medically or behaviorally contraindicated. Animal care was provided by the Laboratory Animal Resources veterinary staff of the respective institutions. The dog colony has a null mutation in intron 1 of the canine α-L-iduronidase gene that results in abnormal mRNA splicing, introduces a premature termination codon, and completely eliminates IDUA protein expression [35]. The four dogs were neither tolerized nor treated with IV rhIDUA, and had received six monthly intra-articular rhIDUA injections as previously reported [36].

Mouse-related protocols were approved by the University of California San Diego IACUC Animal Subjects Committee (IACUC #S99127). The MPS I (Idua-/-) mouse colony was housed, maintained, and veterinarian-supervised at The University of California San Diego, an AAALAC accredited facility. The colony, which is on the C57BL/6 background, was originally obtained from the Jackson Laboratories (B6.129-Iduatm1Clk/J) and was bred locally for 9 generations. Idua genotyping was performed as per S1 Appendix. Mice utilized for this study were fed standard chow and were neither tolerized nor treated with any form of rhIDUA.

Tissue collection and euthanasia

At 18 months of age, dogs were euthanized with a 1 cc / 10 lb dose of euthasol and 1 cm rings from the ascending aorta, descending aorta proximal to the renal arteries, and common carotid arteries were immediately obtained post-mortem. Arterial rings were processed as follows: 1) one section from each site was embedded in a cryomold with OCT (Tissue-Tek CRYO-OCT Compound, ThermoFisher Scientific, Waltham, MA), frozen and stored at -80°C, 2) one section placed in TRIzol solution (Life Technologies, Grand Island, NY) for subsequent RNA extraction, 3) and one section frozen and stored at -80°C for subsequent Western blotting.

At 9–12 months of age, female MPS I (mean age, 9.4 months; n = 8) and age-matched wild-type (mean age, 10.2 months n = 3) mice were euthanized with a combination of Isoflurane inhalation (Abbott, North Chicago, IL), cervical dislocation, and exsanguination. Heart and aorta collection was performed as per Daugherty and Whitman, and summarized as follows: after exsanguination, the right atrium was incised and the animal was perfused with 20 mL cold phosphate buffered saline (PBS) through the left ventricle [37]. The abdominal viscera...
were removed and the heart and aorta were carefully dissected en bloc to the iliac bifurcation. The heart was cut from the ascending aorta approximately 5 mm from the point of emergence from the left ventricle, embedded in a cryomold with OCT, and stored frozen at -80°C.

**RNA isolation, amplification, and labeling**

RNA isolation from canine aortic specimens was performed using the TRIzol RNA Purification System (Life Technologies, Grand Island, NY). RNA concentration and quality, as well as dye incorporation efficiency, was checked with an ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and the Agilent Bioanalyzer 2100. A total of 100 ng RNA was used as initial starting template, which was labeled with Cy-3 or Cy-5 cytidine 5'-triphosphate using the low input fluorescent linear amplification kit (Agilent, Santa Clara, CA).

**Microarray hybridizations and data analysis**

Comparisons of canine arterial gene expression between control and untreated MPS animals were conducted with a canine-specific microarray covering 43,803 probes (Agilent G2519F 4x44k, Santa Clara, CA), for a total of four comparison groups: MPS ascending aorta vs. control ascending aorta, MPS descending aorta vs. control descending aorta, MPS carotid artery vs. control carotid artery, and finally pooled MPS artery (ascending aorta, descending aorta, carotid artery) vs. pooled control artery. Each comparison used four pairs of MPS vs corresponding age- and gender-matched animals to produce four biologic replicates.

**Bioinformatic analysis**

The data was analyzed by the WGCNA package in R 3.0.2, a package we have used previously to analyze large datasets and identify novel genomic targets [38–41]. Co-expression networks were formed on genes with similar behavior. Trait information (e.g. ascending aorta, carotid artery, and descending aorta) and Pearson correlation data were obtained.

Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 was used to functionally annotate the genes that clustered together [42,43]. DAVID’s functional annotation tool allows for pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG), gene ontology annotation for biological processes, molecular function, and cellular components, assessment of transcription factor binding sites, and identification of tissues matching the gene clusters. All relationships determined had corresponding statistics within DAVID. The functional annotations presented here had a p-value \( \leq 0.05 \) with Bonferroni correction.

Network analysis was performed using two methods. The first was WGCNA to identify networks without any prior knowledge. This enabled discovery of novel nodes and edges that could be visualized using Cytoscape version 3.1.1 [44]. The second was with GeneMania (a plugin available in Cytoscape) to examine connections based on prior evidence including co-expression, shared protein domains, pathway, physical interactions, genetic interactions, and co-localization [39, 45].

**Western blotting**

Flash frozen sections of harvested ascending and descending aorta that had been harvested from homozygous MPS I and heterozygous carrier dogs and stored at -80°C were thawed on ice. Sections of approximately 30 to 50 mg of each tissue sample were cut and mixed in a 1:3 weight to volume ratio with lysis buffer containing 4% SDS, EDTA, a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktails (P0044 and
P5726, Sigma-Aldrich, St. Louis, MO). Standard Dounce homogenization was performed while each tissue sample was kept on ice in a 4°C room. After homogenization, each sample was sonicated in a 4°C water bath for 1 hour and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and centrifuged again at 10,000 rpm for 10 minutes. This supernatant was collected and total protein content of each sample was determined with a standard colorimetric assay (Bio-Rad, Philadelphia, PA) converting absorbance measured as optical density units at 595 nm (Shimadzu BioSpec 1601 Double Beam UV-Visible Spectrophotometer, Shimadzu Scientific Instruments, Columbia, MD) to protein concentration using bovine serum albumin as a standard.

Samples were diluted to a final concentration of 4 μg/μL by addition of the appropriate volume of a standard Laemmli loading buffer. These were then heated to 95°C for 20 minutes prior to loading onto a gel (Novex Tris-Glycine 4–20% Mini Protein Gel, Life Technologies, Grand Island, NY). Each well was loaded with 20 μg of total protein and a protein ladder covering the size range from 10 to 180 kDa (PageRuler Prestained Protein Ladder, Life Technologies, Grand Island, NY) was loaded in one well of each gel for reference. Separation was performed via 4–20% Tris-Glycine SDS-PAGE followed by transfer to a nitrocellulose membrane (BioTrace NT nitrocellulose transfer membrane, Pall Corp., Port Washington, NY).

Standard immunoblotting was then performed with mouse monoclonal anti-human clusterin alpha chain (1:1,000 or 1 ng/μL; EMD Millipore, Billerica, MA) diluted in 2.5% nonfat milk and incubated overnight at 4°C. After washing, the membrane was incubated for one hour at room temperature with goat anti-mouse IgG HRP conjugated secondary antibody (1:50,000; Southern Biotechnology, Birmingham, AL, USA) diluted in 2.5% nonfat milk, and then detected with Immobilon Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA). Membranes were then stripped, washed and re-probed with mouse monoclonal anti-human smooth muscle alpha-actin (1:10,000; Dako North America, Inc., Carpinteria, CA) diluted in 2.5% nonfat milk. After washing, the membrane was incubated for one hour at room temperature with goat anti-mouse IgG HRP conjugated secondary antibody (1:100,000; Southern Biotechnology, Birmingham, AL) diluted in 2.5% nonfat milk, and then detected with Immobilon Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA).

Western blot band intensities were quantified using ImageJ. After conversion of Western blot images to black and white and subtraction of background, the signal intensities of clusterin bands were subsequently measured and normalized to signal intensities of corresponding α-actin bands. The ratio of mean normalized MPS I clusterin intensity to mean heterozygote clusterin intensity was calculated to determine fold-change expression in ascending and descending aorta homogenates.

Canine aorta immunohistochemistry

Transverse cuts through each OCT-embedded tissue sample were taken with a cryostat at -20°C to produce 12 micron-thick cross-sectional slices that were mounted on glass slides. Slide mounted tissue sections were covered with neutral PBS at room temperature for twenty minutes, rinsed, and then fixed with 4% paraformaldehyde in PBS for 20 minutes. Slides were washed in PBS and then each tissue section was blocked with hydrogen peroxide for 20 minutes. After washing in PBS each tissue section was then blocked with 5% normal goat serum in PBS for one hour at room temperature. Tissue sections were then incubated overnight at 4°C with mouse monoclonal anti-human clusterin alpha chain antibody (1:2,500 or 0.4 ng/μL; EMD Millipore, Billerica, MA) diluted in 3% BSA in neutral PBS, mouse monoclonal anti-human smooth muscle alpha actin antibody (1:2,500; Dako North America, Inc., Carpinteria, CA) diluted in 3% BSA in neutral PBS, or 3% BSA in neutral PBS alone. Detection of specific
staining was performed with an anti-mouse HRP-conjugated secondary antibody and diaminobenzidine (DAB) substrate-chromogen (EXPOSE Mouse and Rabbit HRP/DAB Detection Kit, Abcam, Cambridge, MA) according to manufacturer’s protocol. Tissue sections were counterstained with hematoxylin prior to visualization. Cover slips were applied to each slide and standard bright field optical microscopy was performed. In the transverse aorta sections positive (DAB) signal appears brown while the counterstain appears blue.

Murine aorta preparation and immunohistochemistry

6 micron-thick cross-sections of heart and aorta were obtained from OCT-embedded samples with a cryostat at -20°C, mounted on glass slides, dried at room temperature for 30 minutes and then fixed in fresh 10% neutral buffered formalin (Fisher #SF94-4, Waltham, MA) for 15 minutes. Slides were washed 3 times in PBS with Tween-20® (PBST). Endogenous peroxidases were blocked with a 0.03% solution of hydrogen peroxide in PBST; then washed another 3 times in PBST. Slides were overlaid with 1% BSA in PBST (Sigma #A4503, St Louis, MO) for 10 minutes, endogenous biotin blocked with Vector Labs #SP-2001 kit, and washed another 3 times in PBST. Slides were then overlaid with 1% BSA in PBST and polyclonal goat anti-mouse clusterin alpha chain (1:100; Santa Cruz #SC-6420, Dallas, TX), incubated at room temperature for 1 hour, and washed 3 times in PBST. Secondary staining was accomplished with biotinylated donkey anti-goat antibody (1:500; Jackson ImmunoResearch Laboratories #805-065-180, West Grove, PA) incubated 30 minutes at room temperature, then washed 3 times in PBST. Slides were then incubated with streptavidin-HRP (1:500; Jackson ImmunoResearch Laboratories #016-030-084, West Grove, PA) for 30 minutes, rinsed, then developed with 3-amino-9-ethylcarbazole chromogen (Vector Labs #SK-4200, Burlingame, CA) for 10 minutes. Slides were counterstained in Mayer’s Hematoxylin (Sigma #MHS16, St Louis, MO) for 1 minute, washed in PBST, air dried, and cover slips applied with aqueous mounting media (Vector Labs #H-5501, Burlingame, CA).

Results

Microarray was performed on IDUA-/- and control (IDUA+/-) canine arterial samples obtained from common carotid artery, ascending aorta, and descending aorta. Data was initially analyzed using principal component analysis (PCA). Two clusters were visualized, clearly differentiating the IDUA-/- arterial expression patterns from the control patterns (Fig 1A). Dendrograms and trait heatmaps were also made using the adjacency matrix technique (S1 Fig). Using both of these dimension reduction methods, it can easily be determined if the samples cluster together or if there are outliers. Again, two main clusters were observed, one for IDUA-/- and the other for control. In addition, expression patterns from different animals at the same arterial sites demonstrated clustering according to IDUA genotype. In other words, carotid artery expression in the MPS I animals clustered together and was distinct from both MPS I ascending aorta expression and descending aorta expression patterns.

Data was subsequently analyzed using whole genome correlation network analysis (WGCNA) to identify specific modules that related back to the MPS I trait. Use of this method loosens the stringency in the analysis, since groups of genes are examined together allows a significance matrix to be made based on the networks formed [46]. Genes were further categorized based on fold-change and additional statistical tests within each module. The lists of probes/gene 2-fold or more upregulated or downregulated compared to control across modules can be found in Tables 1 and 2, respectively; full listings are included in S1 and S2 Tables. Subsequently, a dendrogram was made to represent the modules formed with the Pearson correlations for each trait shown (Fig 1B). There were ten total modules that formed (S2 Fig).
Specific attention was paid to the turquoise and blue modules because those were the most statistically significant modules representing genes consistently upregulated in all three sampled vascular segments of the IDUA-/- dogs (upper panel, Fig 1C), or genes consistently downregulated in all three sampled vascular segments of the IDUA-/- dogs (lower panel, Fig 1C). Further downstream analysis with a volcano plot to identify the top targets within the turquoise and blue modules is seen in S3 Fig.

The pathways significantly implicated by the upregulated gene module (turquoise; Fig 1 D) include antigen processing and presentation, graft-versus-host disease, proteasomal function, and lysosomal function. E. Genes significantly downregulated in IDUA-/- canine arteries are primarily related to cellular adhesion and the cytoskeleton (placed into the dilated cardiomyopathy, focal adhesion, arrhythmogenic right ventricular cardiomyopathy KEGG pathways).

Fig 1. Microarray Results From IDUA-/- Canine Arterial Samples. A. Principal component analysis of IDUA-/- canine arteries compared to control canine arteries clearly shows the formation of two sample clusters separating IDUA-/- and control expression. B. WGCNA dendrogram on the microarray samples showing different modules related to the Pearson correlation of the IDUA-/- and control samples. C. Heatmaps of the two major modules (turquoise and blue) formed by WGCNA showing genes upregulated (top panel) or downregulated (bottom panel) by IDUA deficiency. D. Genes significantly upregulated in IDUA-/- canine arteries include those related to immunity / inflammation (KEGG pathways: antigen processing and presentation, graft-versus-host disease), proteasomal function, and lysosomal function. E. Genes significantly downregulated in IDUA-/- canine arteries are primarily related to cellular adhesion and the cytoskeleton (placed into the dilated cardiomyopathy, focal adhesion, arrhythmogenic right ventricular cardiomyopathy KEGG pathways).

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Table 1. Upregulated Genes in IDUA<sup>−/−</sup> Canine Arteries. The 50 upregulated genes with significant (p-value $\leq 0.05$) and greater than 2-fold level of upregulation in IDUA<sup>−/−</sup> canine arteries, compared to controls. Note that some genes are represented more than once due to existence of multiple mRNA probes per gene. For the full list of significantly upregulated genes, please refer to S1 Table.

| Fold Change | Gene Symbol | Systematic Name | Description |
|-------------|-------------|-----------------|-------------|
| 27.126      | GPNMB       | XM_858105       | PREDICTED: Canis familiaris similar to glycoprotein (transmembrane) nmb isoform b precursor, transcript variant 3 (LOC482355), mRNA [XM_858105] |
| 20.245      | GPNMB       | XM_858105       | PREDICTED: Canis familiaris similar to glycoprotein (transmembrane) nmb isoform b precursor, transcript variant 3 (LOC482355), mRNA [XM_858105] |
| 12.432      | ENSCAFT00000039657 | ENSCAFT00000039657 | Osteopontin (Fragment) |
| 11.926      | CSTB        | XM_535601       | PREDICTED: Canis familiaris similar to Cystatin B |
| 11.566      | ENSCAFT00000039661 | ENSCAFT00000039661 | Osteopontin (Fragment) |
| 10.506      | DLA-DRA1    | ENSCAFT0000001254 | MHC class II DR alpha chain |
| 10.220      | CLU         | NM_001003370    | Canis lupus familiaris clusterin (CLU) |
| 9.595       | CLU         | NM_001003370    | Canis lupus familiaris clusterin (CLU) |
| 9.464       | CTSK        | ENSCAFT00000035613 | Cathepsin K precursor |
| 8.985       | TC71707     | TC71707         | Q49RE5_CANFA (Q49RE5) Cytochrome b, partial (35%) |
| 7.766       | CSTB        | XM_535601       | PREDICTED: Canis familiaris similar to Cystatin B |
| 7.587       | LOC479746   | XM_536874       | PREDICTED: Canis familiaris similar to Ferritin light chain 2 |
| 7.022       | TC57368     | TC57368         | HUMPLPLSTN2 L-plastin (Homo sapiens) |
| 6.975       | GPNMB       | XM_858105       | PREDICTED: Canis familiaris similar to glycoprotein (transmembrane) nmb isoform b precursor, transcript variant 3 (LOC482355), mRNA [XM_858105] |
| 6.968       | CLU         | NM_001003370    | Canis lupus familiaris clusterin (CLU) |
| 6.947       | CD86        | NM_001003146    | Canis lupus familiaris CD86 molecule (CD86) |
| 6.769       | DR104862    | DR104862        | Canine cardiovascular system biased cDNA |
| 6.738       | FTL         | NM_001024636    | Canis lupus familiaris ferritin, light polypeptide |
| 6.718       | CTSS        | NM_001002938    | Canis lupus familiaris cathepsin S (CTSS) |
| 6.704       | CLU         | NM_001003370    | Canis lupus familiaris clusterin (CLU) |
| 6.677       | APOE        | XM_847504       | PREDICTED: Canis familiaris apolipoprotein E, transcript variant 2 (APOE) |
| 6.666       | CTSK        | NM_001033996    | Canis lupus familiaris cathepsin K (CTSK) |
| 6.655       | CTSK        | NM_001033996    | Canis lupus familiaris cathepsin K (CTSK) |
| 6.605       | LGMN        | XM_537355       | PREDICTED: Canis familiaris similar to Legumain precursor |
| 6.453       | CTSK        | NM_001033996    | Canis lupus familiaris cathepsin K (CTSK) |
| 6.423       | DLA-DRB1    | NM_001014768    | Canis lupus familiaris MHC class II DLA DRB1 beta chain (DLA-DRB1) |
| 6.193       | FTL         | NM_001024636    | Canis lupus familiaris ferritin, light polypeptide (FTL) |
| 6.178       | CTSK        | NM_001033996    | Canis lupus familiaris cathepsin K (CTSK) |
| 6.055       | TYRP1       | XM_859450       | PREDICTED: Canis familiaris tyrosinase-related protein 1 (TYRP1) |
| 6.026       | CTSK        | NM_001033996    | Canis lupus familiaris cathepsin K (CTSK) |
| 6.013       | BPI         | XM_534417       | PREDICTED: Canis familiaris similar to Bactericidal permeability-increasing protein precursor (BPI) |
| 6.006       | CTSB        | XM_543203       | PREDICTED: Canis familiaris similar to cathepsin B preproprotein |
| 5.833       | TC50899     | TC50899         | Q9N2I5_FELCA (Q9N2I5) CD16 |
| 5.828       | TC62836     | TC62836         | FCU92795 fusin (Felix catus) |
| 5.727       | LGMN        | XM_537355       | PREDICTED: Canis familiaris similar to Legumain precursor |
| 5.680       | DLA-DQB1    | NM_001014381    | Canis lupus familiaris MHC class II, DQ beta 1 (DLA-DQB1) |
| 5.660       | CSGALNACT1  | XM_539946       | PREDICTED: Canis familiaris similar to chondroitin beta1,4 N-acetylgalactosaminyltransferase |
| 5.651       | MT2A        | NM_001003149    | Canis lupus familiaris metallothionein 2A (MT2A) |
| 5.493       | TC51307     | TC51307         | Unknown |
| 5.331       | TGM2        | XM_542991       | PREDICTED: Canis familiaris similar to Protein-glutamine gamma-glutamyltransferase |
| 5.306       | SMOC2       | XM_858080       | PREDICTED: Canis familiaris similar to secreted modular calcium-binding protein 2 |

(Continued)
lysosome. Specific genes from each upregulated pathway are detailed in Table 3. The pathways significantly implicated by the downregulated gene module (blue; Fig 1 E) include dilated cardiomyopathy, focal adhesion, and arrhythmogenic right ventricular cardiomyopathy and are detailed in Table 4. These identified pathways are consistent with pathways identified from prior expression arrays from MPS IIIb mouse brain [26, 30], MPS VII mouse brain [29] and liver [47].

Next, gene networks were constructed, utilizing two methods to identify any potential interactions between genes with perturbed expression. The first was de novo using WGCNA, and the second utilized GeneMania. Signed networks were constructed in WGCNA using an adjacency function that incorporates gene correlation and connectivity across the dataset. GeneMania networks were constructed based on known interactions such as co-expression, shared protein domains, signaling pathways, physical interactions, genetic interactions, and co-localization. The results of the GeneMania-generated network analysis are shown in Fig 2, with correlated upregulated genes in the MPS I dogs in Fig 2A: notable genes exceeding 2-fold expression change in the upregulated network include lysosomal proteases (CTSB, CTSS, LGMN); RRAGD, a GTPase involved with lysosomal energy sensing; immunity-related genes such as CD86, a macrophage-related signaling protein; BPI, a protein that binds bacterial lipopolysaccharide; and CLU, a marker previously identified in inflammatory processes and atherosclerosis. Fig 2B shows the correlated downregulated (reduced 2-fold) gene network in the MPS I dogs. Many of the downregulated genes are smooth muscle cytoskeletal elements, including TCAP (titin-cap), TPN1 (troponymosin), DMD (dystrophin), SMTN (smoothelin), TAGLN (transgelin), MYH11 (smooth muscle myosin heavy chain 11), and CNN1 (smooth muscle calponin 1).

We further examined the expression pattern of clusterin, which was present in the upregulated gene network and the most significantly overexpressed gene (S3 Fig). The clusterin overexpression especially interested us, as it has been shown to be involved in morphologic transformation of vascular smooth muscle cells [48] and present in human atherosclerotic plaques [49]. The MPS I dog aortas contained multiple eccentric plaques that intruded into the lumen, consistent with previous reports of vascular pathology in the model [23]. Importantly, we confirmed that clusterin protein is overexpressed in MPS I ascending (1.3-fold) and descending aortas (1.62-fold) compared to unaffected heterozygous dogs (Fig 3). The clusterin protein is localized primarily within the plaques of the MPS I aortas; little to no clusterin signal was seen in the control aortas (Fig 4). These results provide protein-level confirmation of

| Fold Change | Gene Symbol | Systematic Name | Description |
|-------------|-------------|-----------------|-------------|
| 5.190       | ASAH1       | XM_540012       | PREDICTED: Canis familiaris similar to N-acylsphingosine amidohydrolase (acid ceramidase) |
| 5.152       | APOE        | ENSCAFT0000007432 | Apolipoprotein E (Apo-E) |
| 5.104       | TC54889     | TC54889         | Unknown |
| 5.069       | DLA-DRA1    | NM_001011723    | Canis lupus familiaris MHC class II DR alpha chain (DLA-DRA1) |
| 4.984       | RRAGD       | XM_532231       | PREDICTED: Canis familiaris similar to Ras-related GTP binding D |
| 4.921       | E02824      | E02824          | Canis familiaris cDNA clone LIB30232 006_G04 |
| 4.912       | RRAGD       | XM_532231       | PREDICTED: Canis familiaris similar to Ras-related GTP binding D |
| 4.902       | TYROBP      | XM_533687       | PREDICTED: Canis familiaris similar to TYRO protein tyrosine kinase binding protein isoform 2 precursor |
| 4.882       | FTH1        | ENSCAFT00000025200 | Ferritin heavy chain (EC 1.16.3.1) (Ferritin H subunit) |

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Table 2. Down-regulated Genes in IDUA⁻/⁻ Canine Arteries. The 50 down-regulated genes with significant (p-value ≤ 0.05) and greater than 2-fold level of down-regulation in IDUA⁻/⁻ canine arteries, compared to controls. Again, some genes are represented more than once due to existence of multiple mRNA probes per gene. For the full list of significantly down-regulated genes, please refer to S2 Table.

| Fold Change | Gene Symbol | Systematic Name | Description |
|-------------|-------------|-----------------|-------------|
| 4.948       | TC67209     | TC67209         | HUMRPS17A S17 ribosomal protein (Homo sapiens) |
| 3.970       | LRRK2       | XM_543734       | PREDICTED: Canis familiaris similar to leucine-rich repeat kinase 2 |
| 3.666       | CNN1        | XM_862565       | PREDICTED: Canis familiaris similar to calponin 1, basic, smooth muscle, transcript variant 4 |
| 3.392       | CO611926    | CO611926        | DG9-109j22 DG9-ovary Canis familiaris cDNA 3' |
| 3.309       | SMTN        | XM_860837       | PREDICTED: Canis familiaris similar to smoothelin isoform c |
| 3.218       | NRGN        | XM_536537       | PREDICTED: Canis familiaris similar to neurogranin (LOC479401) |
| 3.212       | CSRP1       | XM_843516       | PREDICTED: Canis familiaris similar to Cysteine and glycine-rich protein 1 |
| 3.157       | TCAP        | XM_858636       | PREDICTED: Canis familiaris similar to Telethonin (Titin cap protein) |
| 3.109       | PPP1R12B    | XM_843384       | PREDICTED: Canis familiaris similar to Protein phosphatase 1 regulatory subunit 12B (Myosin phosphatase targeting subunit 2) |
| 3.018       | DN756599    | DN756599        | GLC-13395 GLGC-LIB0001-cf Canis familiaris Normalized Mixed Tissue cDNA |
| 2.949       | LOC488984   | XM_843847       | PREDICTED: Canis familiaris similar to Actin, alpha skeletal muscle |
| 2.865       | AB012223    | AB012223        | Canis familiaris LINE-1 element ORF2 mRNA |
| 2.855       | ENSCAFT00000034839 | ENSCAFT00000034839 | NADH-ubiquinone oxido-reductase chain 3 |
| 2.836       | PPP1R12B    | XM_843384       | PREDICTED: Canis familiaris similar to Protein phosphatase 1 regulatory subunit 12B (Myosin phosphatase targeting subunit 2) |
| 2.812       | ENSCAFT00000034824 | ENSCAFT00000034824 | NADH-ubiquinone oxido-reductase chain 2 (EC 1.6.5.3) (NADH dehydrogenase subunit 2) |
| 2.812       | BM538907    | BM538907        | hb02c06.g1 Canis cDNA from testes cells Canis familiaris cDNA clone |
| 2.782       | LOC488984   | XM_843847       | PREDICTED: Canis familiaris similar to Actin, alpha skeletal muscle |
| 2.764       | TC55776     | TC55776         | Unknown |
| 2.761       | TAGLN       | XM_850622       | PREDICTED: Canis familiaris similar to transgelin, transcript variant 3 |
| 2.734       | FHL1        | XM_540928       | PREDICTED: Canis familiaris similar to four and a half LIM domains 1 |
| 2.712       | LOC488984   | XM_843847       | PREDICTED: Canis familiaris similar to Actin, alpha skeletal muscle |
| 2.705       | OGN         | XM_848247       | PREDICTED: Canis familiaris similar to Mimecan precursor (Osteoglycin) |
| 2.692       | DMD         | NM_00103343     | Canis lupus familiaris dystrophin (muscular dystrophy, Duchenne and Becker types) |
| 2.673       | TC68286     | TC68286         | Unknown |
| 2.630       | ENSCAFT00000022802 | ENSCAFT00000022802 | Fibronectin (FN) (Fragment) |
| 2.626       | SLC22A3     | XM_533467       | PREDICTED: Canis familiaris similar to solute carrier family 22 member 3 |
| 2.617       | LOC488984   | XM_843847       | PREDICTED: Canis familiaris similar to Actin, alpha skeletal muscle |
| 2.598       | DN879822    | DN879822        | nac29h08.y1 Dog eye eye minus lens and cornea. Unnormalized (nac) |
| 2.594       | PTGS1       | NM_001003023    | Canis lupus familiaris prostaglandin-endoperoxide synthase 1 |
| 2.586       | LOC491592   | XM_548713       | PREDICTED: Canis familiaris similar to ankyrin repeat domain 26 |
| 2.567       | PPPAP2B     | XM_536696       | PREDICTED: Canis familiaris similar to phosphatidic acid phosphatase type 2B |
| 2.526       | PPPAP2B     | XM_536696       | PREDICTED: Canis familiaris similar to phosphatidic acid phosphatase type 2B |
| 2.489       | C1QTNF1     | XM_843609       | PREDICTED: Canis familiaris similar to C1q and tumor necrosis factor related protein 1 |
| 2.481       | TPM1        | ENSCAFT00000026876 | Tropomyosin (Fragment) |
| 2.460       | MYH11       | XM_857511       | PREDICTED: Canis familiaris similar to smooth muscle myosin heavy chain 11 isoform SM1 |
| 2.459       | ADCY5       | M88649          | Canis familiaris adenyly cyclase type V |
| 2.458       | LOC480803   | XM_537918       | PREDICTED: Canis familiaris similar to ankyrin repeat domain 26 |
| 2.429       | TC48376     | TC48376         | MALAT_HUMAN (Q9UHZ2) Metastasis-associated lung adenocarcinoma transcript 1 |
| 2.407       | CF412534    | CF412534        | Canine heart normalized cDNA Library, pBluescript Canis familiaris cDNA clone CH3#080_C09 5' |

(Continued)
clusterin gene overexpression within MPS I arterial vasculature. Additionally, Toll-like receptor 4 (TLR4) protein overexpression and increased phosphorylated STAT1 protein were seen only in canine MPS I arteries (S4 Fig).

Next, we assessed whether adult C57/Bl6 Idua−/− mice had similar cardiovascular findings to the IDUA−/− dogs, especially with regards to aortic histopathology. The mice had grossly evident hypertrophic cardiomyopathy compared to Idua+/− control mice, which is consistent with other assessments of cardiovascular phenotype [23]. While not as prominent as the canine MPS I model, the Idua−/− mice also demonstrated eccentric, sclerotic plaques and thickened proximal aortas compared to control mice. These plaques were visible only near the sinuses of Valsalva, a distribution different from the canine plaques, which were present throughout the ascending and descending aorta. Clusterin protein was absent in the control mice (Fig 5A), and prominently visible in the aortic plaques of the Idua−/− mice (Fig 5B). The full clusterin and smooth muscle actin blots can be viewed in S5 Fig.

Discussion

We performed microarray analyses comparing arterial gene expression of untreated MPS I canines to unaffected heterozygous canines to gain insights into the gene perturbations caused by α-L-iduronidase deficiency and subsequent arterial glycosaminoglycan storage. Our study represents a comprehensive assessment of gene expression in the vasculature of any MPS model system, and identified upregulation of genes related to lysosomes, proteasomes, macrophages, and innate immunity. We also identified downregulation of cytoskeletal genes and calcium channel subunits. Our findings are consistent with results of gene expression studies from non-vascular tissues in differing MPS subtypes. This indicates the possibility that common mechanisms may be responsible for pathogenesis of MPS disease in multiple organ systems. We also identified evidence linking the innate immune system to pathogenesis of MPS I cardiovascular disease, as well as a biomarker for potentially following disease progression and treatment efficacy.

Overexpression of lysosomal hydrolases and housekeeping genes is consistent with the hypothesis that accumulation of primary substrate in lysosomal storage disorders results in a “traffic jam” with secondarily impaired degradation of other lysosomal substrates, and
Table 3. Upregulated Canine Arterial IDUA-/- Genes Sorted by Category. Upregulated arterial genes in IDUA-/- canines fall into categories associated with lysosomal function, proteasomal function, and the immune system (graft-versus-host disease and antigen processing).

| Ontology Category | Gene Symbol | Gene Name | Entrez Gene ID |
|-------------------|-------------|-----------|---------------|
| **LYSOSOMAL FUNCTION** | ATP6V0C | ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c | 479877 |
| | ATP6V0B | ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b | 482528 |
| | ATP6V0D1 | ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1 | 479685 |
| | ATP6V1H | ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H | 486953 |
| | ATP6V0A1 | ATPase, H+ transporting, lysosomal V0 subunit a1 | 607705 |
| | ATP6AP1 | ATPase, H+ transporting, lysosomal accessory protein 1 | 610922 |
| | CD63 | CD63 molecule | 474391 |
| | GM2A | GM2 ganglioside activator | 479324 |
| | NAGA | N-acetylgalactosaminidase, alpha | 481226 |
| | GNPTAB | N-acetylgalactosamine-1-phosphate transferase, alpha and beta subunits | 475443 |
| | NAGPA | N-acetylgalactosamine-1-phosphodiester alpha-N-acetylglucosaminidase | 490019 |
| | NAGLU | N-acetylgalactosaminidase, alpha- | 490965 |
| | ASAH1 | N-acylsphingosine amidohydrolase (acid ceramidase) 1 | 482897 |
| | SGSH | N-sulfogalactosamine sulfotransferase | 403707 |
| | NPC1 | Niemann-Pick disease, type C1 | 403698 |
| | TCIRG1 | T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3 | 483691 |
| | AP3B2 | adaptor-related protein complex 3, beta 2 subunit | 479053 |
| | AP3M1 | adaptor-related protein complex 3, mu 1 subunit | 489052 |
| | AP3S2 | adaptor-related protein complex 3, sigma 2 subunit | 479042 |
| | AP4B1 | adaptor-related protein complex 4, beta 1 subunit | 483125 |
| | ARSA | arylsulfatase A | 474457 |
| | AGA | aspartylglucosaminidase | 475638 |
| | CTSA | cathepsin A | 611146 |
| | CTSB | cathepsin B | 486077 |
| | CTSC | cathepsin C | 403458; 478608 |
| | CTSN | cathepsin D | 483662 |
| | CTSG | cathepsin G | 608543 |
| | CTHH | cathepsin H | 479065 |
| | CTSH | cathepsin K | 608843 |
| | CTSL2 | cathepsin L2 | 403708 |
| | CTSS | cathepsin S | 403400 |
| | CTSZ | cathepsin Z | 611983 |
| | CLTA | clathrin, light chain (Lca) | 474765 |
| | CTNS | cystinosin, nephropathic | 491220 |
| | DNASE2 | deoxyribonuclease II, lysosomal | 476697 |
| | GLB1 | galactosidase, beta 1 | 403873 |
| | GBA | glucosidase, beta; acid (includes glucosylceramidase) | 612206 |
| | GUSB | glucuronidase, beta | 403831 |
| | GGA2 | golgi associated, gamma adaptin ear containing, ARF binding protein 2 | 608555 |
| | HGSNAT | heparan-alpha-glucosaminide N-acetyltransferase | 482833 |
| | HEXA | hexosaminidase A (alpha polypeptide) | 487633 |
| | HEXB | hexosaminidase B (beta polypeptide) | 478100 |
| | LGMN | legumain | 480323 |
| | LIPA | lipase A, lysosomal acid, cholesterol esterase | 610650 |

(Continued)
### Table 3. (Continued)

#### Ontology Category
| Gene Symbol | Gene Name | Entrez Gene ID |
|-------------|-----------|----------------|
| LAPTM5      | lysosomal multispanning membrane protein 5 | 487324 |
| LAPTM4A     | lysosomal protein transmembrane 4 alpha | 475678 |
| LAMP1       | lysosomal-associated membrane protein 1 | 476995 |
| LAMP2       | lysosomal-associated membrane protein 2 | 481037 |
| M6PR        | mannone-6-phosphate receptor (cation dependent) | 477700 |
| MAN2B1      | mannosidase, alpha, class 2B, member 1; similar to UPF0139 protein CGI-140 | 476703; 484932 |
| MANBA       | mannosidase, beta A, lysosomal | 487883 |
| PPT1        | palmitoyl-protein thioesterase 1 | 475316 |
| PPT2        | palmitoyl-protein thioesterase 2 | 474856 |
| PSAP        | prosaposin | 479240 |
| SCARB2      | scavenger receptor class B, member 2 | 478435 |
| NEU1        | sialidase 1 (lysosomal sialidase) | 481717 |
| AP1S2       | similar to Adapter-related protein complex 1 sigma 1B subunit (Sigma-adaptin 1B) | 611468 |
| SLC11A1     | solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 | 478909 |
| SLC17A5     | solute carrier family 17 (anion/sugar transporter), member 5 | 474969 |
| SORT1       | sortilin 1 | 479915 |
| TPP1        | tripeptidyl peptidase I | 485337 |

#### PROTEASOMAL FUNCTION

| Gene Symbol | Gene Name | Entrez Gene ID |
|-------------|-----------|----------------|
| PSMC1       | proteasome (prosome, macropain) 26S subunit, ATPase, 1 | 478703 |
| PSMC2       | proteasome (prosome, macropain) 26S subunit, ATPase, 2 | 475896 |
| PSMC3       | proteasome (prosome, macropain) 26S subunit, ATPase, 3 | 475980 |
| PSMC5       | proteasome (prosome, macropain) 26S subunit, ATPase, 5 | 480478 |
| PSMD11      | proteasome (prosome, macropain) 26S subunit, non-ATPase, 11 | 480610 |
| PSMD14      | proteasome (prosome, macropain) 26S subunit, non-ATPase, 14 | 478765 |
| PSMD2       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 2 | 478654 |
| PSMD3       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 3 | 491018 |
| PSMD6       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 6 | 484700 |
| PSMD8       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 | 476470 |
| PSEME1      | proteasome (prosome, macropain) activator subunit 1 (PA28 alpha) | 480256 |
| PSEME2      | proteasome (prosome, macropain) activator subunit 2 (PA28 beta) | 480258 |
| PSEME4      | proteasome (prosome, macropain) activator subunit 4 | 474594 |
| PSEMA4      | proteasome (prosome, macropain) subunit, alpha type, 4 | 475132 |
| PSEMA5      | proteasome (prosome, macropain) subunit, alpha type, 5 | 490123 |
| PSEMA6      | proteasome (prosome, macropain) subunit, alpha type, 6 | 480290 |
| PSEMA7      | proteasome (prosome, macropain) subunit, alpha type, 7 | 404305 |
| PSEMB1      | proteasome (prosome, macropain) subunit, beta type, 1 | 475040 |
| PSEMB10     | proteasome (prosome, macropain) subunit, beta type, 10 | 489749 |
| PSEMB2      | proteasome (prosome, macropain) subunit, beta type, 2 | 475338 |
| PSEMB3      | proteasome (prosome, macropain) subunit, beta type, 3 | 474987; 480537 |
| PSEMB4      | proteasome (prosome, macropain) subunit, beta type, 4 | 475848 |
| PSEMB5      | proteasome (prosome, macropain) subunit, beta type, 5 | 480246 |
| PSEMB8      | proteasome (prosome, macropain) subunit, beta type, 8 | 474865 |
| PSEMB9      | proteasome (prosome, macropain) subunit, beta type, 9 | 474867 |
| POMP        | proteasome maturation protein | 477325 |
| PSEMA1      | similar to Proteasome subunit alpha type 1 (Proteasome component C2) | 476867; 608388; 610188; 612667; 613000 |

(Continued)
Table 3. (Continued)

| Ontology Category       | Gene Symbol | Gene Name                                      | Entrez Gene ID          |
|-------------------------|-------------|-----------------------------------------------|-------------------------|
| PSMA3                   | CD80        | CD80 molecule                                 | 403765                  |
|                         | CD86        | CD86 molecule                                 | 403764                  |
|                         | DLA-12 / DLA-64 | MHC class I DLA-12; HMC class I DLA-64       | 474838; 541592          |
|                         | DLA88       | MHC class I DLA-88                            | 474836                  |
|                         | DLA-DRB1    | MHC class II DLA DRB1 beta chain              | 474860                  |
|                         | DLA-DRA1    | MHC class II DR alpha chain                   | 481731                  |
|                         | DLA-79      | MHC class lb                                  | 483594                  |
|                         | IL1A        | interleukin 1, alpha                          | 403782                  |
|                         | IL1B        | interleukin 1, beta                           | 403974                  |
|                         | IL2         | interleukin 2                                 | 403989                  |
|                         | IL6         | interleukin 6 (interferon, beta 2)            | 403985                  |
|                         | HLA-DMA     | major histocompatibility complex, class II, DM alpha | 481732                  |
|                         | HLA-DOB     | major histocompatibility complex, class II, DO beta | 607786                  |
|                         | DLA-DQA1    | major histocompatibility complex, class II, DO alpha 1 | 474861                  |
|                         | DLA-DQB1    | major histocompatibility complex, class II, DO beta 1 | 474862                  |
| "ANTIGEN PROCESSING"    | DLA-12 / DLA-64 | MHC class I DLA-12; HMC class I DLA-64       | 474838; 541592          |
|                         | DLA88       | MHC class I DLA-88                            | 474836                  |
|                         | DLA-DRB1    | MHC class II DLA DRB1 beta chain              | 474860                  |
|                         | DLA-DRA1    | MHC class II DR alpha chain                   | 481731                  |
|                         | DLA-79      | MHC class lb                                  | 483594                  |
|                         | TAPBP       | TAP binding protein (tapasin)                 | 481740                  |
|                         | B2M         | beta-2-microglobulin                          | 478284                  |
|                         | CANX        | calnexin                                      | 403908                  |
|                         | CTSB        | cathepsin B                                   | 486077                  |
|                         | CTSS        | cathepsin S                                   | 403400                  |
|                         | HSPA1L      | heat shock 70kDa protein 1-like               | 474850                  |
|                         | HSP70       | heat shock protein 70                         | 403612                  |
|                         | HSPA4       | heat shock protein Apg-2                      | 474880                  |
|                         | KLRD1       | killer cell lectin-like receptor subfamily D, member 1 | 611360                  |
|                         | LGMN        | legumain                                      | 480232                  |
|                         | HLA-DMA     | major histocompatibility complex, class II, DM alpha | 481732                  |
|                         | HLA-DOB     | major histocompatibility complex, class II, DO beta | 607786                  |
|                         | DLA-DQA1    | major histocompatibility complex, class II, DO alpha 1 | 474861                  |
|                         | DLA-DQB1    | major histocompatibility complex, class II, DO beta 1 | 474862                  |
|                         | NFYB        | nuclear transcription factor Y, beta          | 475450                  |
|                         | NFYC        | nuclear transcription factor Y, gamma         | 475312                  |
|                         | PSME1       | proteasome (prosome, macropain) activator subunit 1 (PA28 alpha) | 480256                  |
|                         | PSME2       | proteasome (prosome, macropain) activator subunit 2 (PA28 beta) | 480258                  |
|                         | RXANK       | regulatory factor X-associated ankyrin-containing protein | 476662                  |
|                         | LOC480726   | similar to 78 kDa glucose-regulated protein precursor (Endoplasmic reticulum luminal Ca(2+) binding protein grp78) | 480726                  |
subsequent efforts by the cell to alleviate the secondary storage [50]. Our findings of cathepsin protease overexpression are consistent with aortic cathepsin B, D, L, and S overexpression from the mouse and canine models of MPS I and VII [31, 33, 34]. In addition, overexpression of lysosomal membrane proteins and enzymes has been observed in murine MPS VII liver [47] and brain [29]. An additional effect of the lysosomal traffic jam is impairment in organelle and protein degradation via a lysosome-dependent pathway known as autophagy [51]. Since the ubiquitin-proteasome system (UPS) is another prominent mechanism for recycling of accumulated proteins, the observed upregulation of proteasomal genes may indicate diversion of protein catabolism towards the UPS.

Downregulation of cellular adhesion and cytoskeletal genes (labeled as "Dilated Cardiomyopathy," "Focal Adhesion," and "Arrhythmogenic Right Ventricular Cardiomyopathy" in our study) has also been seen in MPS IIIb mouse brains [30]. These results support data suggesting that excess HS oligosaccharides impair function of cellular adhesion molecules and disrupt normal polarization and orientation of cultured MPSIIIb mouse astrocytes or neural stem cells [52].

The generalized upregulation of macrophage and immunity-related genes observed in the MPS I arteries indicate that inflammation may play a prominent role in the pathogenesis of MPS I cardiovascular disease. This immune-associated gene overexpression was also seen in several expression studies in MPS IIIb mice brains [26, 30] and is consistent with neuroinflammation observed in murine MPS IIIb and other neuropathic lysosomal storage disorders, including Sandhoff Disease and MPS I [53, 54]. While exploring potential etiologies of MPS I cardiovascular disease, significant overexpression of the Toll-like receptor 4 gene, in addition to numerous cathepsin proteases and matrix metalloproteinases was observed [34]. When MPS VII / cathepsin S and MPS VII / MMP-12 double knockout mice continued to develop arterial elastin degradation and aortic root dilatation, it was concluded that other proteases or inflammatory signals transduced by TLR4 activation could be responsible for the MPS VII cardiovascular phenotype [31].

TLR4 is a transmembrane protein that belongs to a family of proteins known as Pattern Recognition Receptors (PRRs). PRRs are expressed by cells of the innate immune system, bind foreign molecules that are typically associated with infections (pathogen-associated molecular patterns or PAMPs) or molecules released by damaged cells (damage-associated molecular patterns or DAMPs), and activate an immune response [55]. Lipopolysaccharide, the canonical PAMP for bacteria, activates TLR4 signaling. Acting via the MyD88 adaptor and STAT1

| Ontology Category | LOC | Similar to |
|-------------------|-----|------------|
| LOC476669         |     | similar to Gamma-interferon inducible lysosomal thiol reductase precursor (Gamma-interferon-inducible protein IP-30) |
| LOC479329         |     | similar to HLA class II histocompatibility antigen, gamma chain (HLA-DR antigens associated invariant chain) (Ia antigen-associated invariant chain) |
| LOC607470         |     | similar to Heat shock protein HSP 90-alpha (HSP 86) |
| LOC490008         |     | similar to MHC class II transactivator |
| HSPA8             |     | similar to heat shock 70kDa protein 8 isoform 2; heat shock 70kDa protein 8; similar to Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8); similar to heat shock protein 8 |
| LOC480438         |     | similar to heat shock protein 1, alpha |
| LOC608885         |     | similar to heat shock protein 8 |

Table 3. (Continued)
### Table 4. Down-regulated Canine Arterial IDUA-/- Genes Sorted by Category

Down-regulated arterial genes in IDUA-/- dogs fall into categories associated with cytoskeletal proteins, cellular adhesion, and ion channels (termed arrhythmogenic right ventricular cardiomyopathy, focal adhesion, and dilated cardiomyopathy).

#### Ontology Category

**ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY**

| Systematic Name | Gene Name                                      | Entrez Gene ID |
|-----------------|------------------------------------------------|----------------|
| ACTB            | actin, beta                                   | 610787         |
| ACTN1           | actinin, alpha 1                              | 480369         |
| ACTN2           | actinin, alpha 2                              | 479191         |
| ACTN4           | actinin, alpha 4                              | 484526         |
| LOC442937       | beta-actin                                    | 442937         |
| CACNA1S         | calcium channel, voltage-dependent, L type, alpha 1S subunit | 490244         |
| CACNB1          | calcium channel, voltage-dependent, beta 1 subunit | 491030         |
| CACNB2          | calcium channel, voltage-dependent, beta 2 subunit | 487113         |
| CACNB4          | calcium channel, voltage-dependent, beta 4 subunit | 609361         |
| CTNNA1          | catenin (cadherin-associated protein), alpha 2 | 483088         |
| CTNNA3          | catenin (cadherin-associated protein), alpha 3 | 489008         |
| DES             | desmin                                        | 497091         |
| DSC2            | desmocollin 2                                 | 403860         |
| DMD             | dystrophin (muscular dystrophy, Duchenne and Becker types) | 606758         |
| GJA1            | gap junction protein, alpha 1, 43kDa          | 403418         |
| ITGA5           | integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | 486493         |
| ITGA6           | integrin, alpha 6                             | 478800         |
| ITGA8           | integrin, alpha 8                             | 487119         |
| ITGA9           | integrin, alpha 9                             | 477021         |
| ITGB4           | integrin, beta 4                              | 483318         |
| ITGB8           | integrin, beta 8                              | 475253         |
| RYR2            | ryanodine receptor 2 (cardiac)                | 403615         |
| SGCG            | sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein) | 486043         |
| LOC492249       | similar to Emerin                             | 492249         |

**DILATED CARDIOMYOPATHY**

| Systematic Name | Gene Name                                      | Entrez Gene ID |
|-----------------|------------------------------------------------|----------------|
| GNAS            | GNAS complex locus                            | 403943         |
| ACTB            | actin, beta                                   | 610787         |
| ADCY2           | adenylate cyclase 2 (brain)                   | 478624         |
| ADCY5           | adenylate cyclase 5                           | 403859         |
| ADCY9           | adenylate cyclase 9                           | 490031         |
| LOC442937       | beta-actin                                    | 442937         |
| CACNA1S         | calcium channel, voltage-dependent, L type, alpha 1S subunit | 490244         |
| CACNB1          | calcium channel, voltage-dependent, beta 1 subunit | 491030         |
| CACNB2          | calcium channel, voltage-dependent, beta 2 subunit | 487113         |
| CACNB4          | calcium channel, voltage-dependent, beta 4 subunit | 609361         |
| DES             | desmin                                        | 497091         |
| DMD             | dystrophin (muscular dystrophy, Duchenne and Becker types) | 606758         |
| ITGA5           | integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | 486493         |

(Continued)
| Ontology Category | Systematic Name | Gene Name | Entrez Gene ID |
|-------------------|----------------|-----------|---------------|
| ITGA6 integrin, alpha 6 | RAP1A, member of RAS oncogene family | RAP1A | 483225 |
| ITGA8 integrin, alpha 8 | Rap guanine nucleotide exchange factor (GEF) 1 | RAPGEF1 | 491286 |
| ITGA9 integrin, alpha 9 | Rho GTPase activating protein 5 | ARHGAP5 | 490642 |
| ITGB4 integrin, beta 4 | Rho-associated, coiled-coil containing protein kinase 1 | ROCK1 | 480181 |
| ITGB8 integrin, beta 8 | actin, beta | ACTB | 610787 |
| PLN phospholamban | actinin, alpha 1 | ACTN1 | 480369 |
| RYR2 ryanodine receptor 2 (cardiac) | actinin, alpha 2 | ACTN2 | 479191 |
| SGCGL sarcoglycan, gamma | actinin, alpha 4 | ACTN4 | 484526 |
| LOC492249 similar to Emerin | beta-actin | LOC442937 | 442937 |
| TTN titin | caveolin 1, caveolae protein, 22kDa | CAV1 | 403980 |
| TPM3 tropomyosin 3, tropomyosin 1 (alpha); similar to tropomyosin 1 alpha chain isoform 4 | filament C, gamma | FLNC | 482266 |
| TNF tumor necrosis factor (TNF superfamily, member 2) | integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | ITGA5 | 486493 |
| *FOCAL ADHESION* | integrin, alpha 6 | ITGA6 | 478800 |
| | integrin, alpha 8 | ITGA8 | 487119 |
| | integrin, alpha 9 | ITGA9 | 477021 |
| | integrin, beta 4 | ITGB4 | 483318 |
| | integrin, beta 8 | ITGB8 | 475253 |
| | laminin, alpha 3 | LAMA3 | 480173 |
| | laminin, beta 2 (laminin S) | LAMB2 | 476626 |
| | met proto-oncogene (hepatocyte growth factor receptor) | MET | 403438 |
| | mitogen-activated protein kinase 8 | MAPK8 | 477746 |
| | myosin light chain kinase | MYLK | 488012 |
| | myosin light chain kinase 2 | MYLKC | 477187 |
protein phosphorylation, activated TLR4 results in nuclear translocation of the transcription factor NF-κB and subsequent mRNA expression of proinflammatory cytokines.

Following the discovery that heparan sulfate, but neither chondroitin sulfate nor heparin, activates TLR4 signaling in dendritic cells and macrophages, evidence has been accumulating that heparan sulfate may play a role in the pathogenesis of MPS neurologic and orthopedic disease via TLR4-induced inflammation [56–58]. Wild-type mouse microglia respond in a dose-dependent fashion to HS-oligosaccharides isolated from MPS IIIB patients with overexpression of macrophage inflammatory protein 1α (Mip1a or Ccl3) and interleukin 1β mRNAs. There was significantly reduced cytokine mRNA expression when the same HS-oligosaccharides were administered to Tlr4−/− or MyD88−/− mouse microglia. MPS IIIb (Naglu−/−) mouse brains overexpress Mip1a mRNA as early as postnatal day 10 with concomitant microglial activation and neuroinflammation. Naglu−/− Tlr4−/− or Naglu−/− MyD88−/− mouse microglia. MPS IIIb (Naglu−/−) mouse brains overexpress Mip1a and demonstrated delayed neuroinflammation [33]. Similar neuroinflammatory findings have been reported in MPS IIIa, IIIb, and IIIc mice, all of which accumulate HS [28].

Our results suggest that HS–TLR4 mediated, monocyte/macrophage-induced inflammation contributes to the pathogenesis of cardiovascular disease in MPS I. Among the genes with highest overexpression in MPS I arteries was CD86 which, in conjunction with another overexpressed gene CD80, encode proteins found on the cell surface of activated monocytes. Other immunity-related genes with observed overexpression were members of the major histocompatibility complex II families, which are involved in antigen presentation and are found primarily on monocytes, macrophages, and dendritic cells. In addition, overexpression of the inflammatory cytokines interleukin-1α, interleukin-1β, interleukin 2, and interleukin 6 were observed. We also identified MPS I arterial overexpression of TLR4 protein and evidence of its activation via increased levels of phosphorylated STAT1.

Heparan sulfate-mediated inflammation cannot be the sole etiology of MPS cardiovascular disease pathogenesis. Patients who have MPS type VI store only DS GAG but often develop mitral or aortic valve dysfunction severe enough to necessitate surgical valve replacement, and

Table 4. (Continued)

| Ontology Category | Gene Name | Description | Gene ID |
|-------------------|-----------|-------------|---------|
| PAK4              | p21 protein (Cdc42/Rac)-activated kinase 4 | 484513 |
| PIK3CA            | phosphoinositide-3-kinase, catalytic, alpha polypeptide | 488084 |
| PIK3R2            | phosphoinositide-3-kinase, regulatory subunit 2 (beta) | 609956 |
| PGF               | placental growth factor | 611916 |
| PDGFD             | platelet derived growth factor D | 479460 |
| PDGFRB            | platelet-derived growth factor receptor, beta polypeptide | 442985 |
| PPP1CB            | protein phosphatase 1, catalytic subunit, beta isoform | 403558 |
| PPP1CC            | protein phosphatase 1, catalytic subunit, gamma isoform | 403557 |
| PPP1R12A          | protein phosphatase 1, regulatory (inhibitor) subunit 12A | 475411 |
| RHOA              | ras homolog gene family, member A | 403954 |
| LOC479252         | similar to Vinculin (Metavinculin) | 479252 |
| LOC479790         | similar to mitogen activated protein kinase 3 | 479790 |
| TLN1              | talin 1 | 474759 |
| TNC               | tenasin C | 481689 |
| AKT2              | v-akt murine thymoma viral oncogene homolog 2 | 449021 |
| CRKL              | v-crk sarcoma virus CT10 oncogene homolog (avian)-like | 608125 |

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also demonstrate coronary artery intimal thickening with arterial stiffness \[2, 19, 59\]. Although the effects of DS GAG on TLR4 in MPS have not been directly investigated, there are studies that indicate it may also serve as a PAMP for TLR4 and promote inflammation like HS GAGs \[60, 61\]. Post-mortem studies of several MPS IVa patients, who store neither HS nor DS but...
accumulate keratan and chondroitin sulfate GAGs, have also demonstrated coronary sclerosis or aortic intimal thickening with macrophage infiltration and elastin fibril disruption [62, 63].

Fig 3. Western Blot of Clusterin and Smooth Muscle Actin in Canine Aortas. Clusterin is predicted to appear as a thicker band between 35–39 kDa owing to differential glycosylation. Alpha smooth muscle actin is predicted to appear at approximately 42 kDa. 20 μg of total protein was loaded in each lane. The upper set is from ascending aorta and the lower set is from descending aorta. Normalized mean clusterin band intensities from descending and ascending aortas are 1.62 and 1.3 fold greater in MPS I animals compared to unaffected animals.

Fig 4. Canine Arterial Clusterin Immunohistochemistry. The MPS I aorta (right panel) has an overall increase in clusterin signal compared to unaffected aorta (left panel), and also contains an eccentric plaque (arrowhead) that stains strongly for clusterin.
As our primary focus was canine MPS I arterial disease, mRNA and protein quantification of murine aorta clusterin or pSTAT1 were not performed. Despite this limitation, our study provides evidence from both canine and murine models confirming clusterin protein overexpression within MPS I arterial lesions. We believe that clusterin may have utility as an *in vivo* biomarker of MPS I cardiovascular disease for several reasons. First, overexpression of clusterin has been observed in many inflammatory conditions, including human atheromatous atherosclerosis, where it is observed only in vascular smooth muscle cells and stroma of atheromas and not in normal aorta [64, 65]. Second, while the effects of clusterin overexpression on human cardiovascular disease are unclear and may be pro-atherosclerotic [66] or protective [67, 68], clusterin serum levels correlate with severity of coronary artery stenosis and are highest during active myocardial infarction [69, 70]. Although lipid-mediated atheroma formation and atherosclerosis appear to proceed via pathways distinct from what occurs in MPS I, the roles of macrophages, inflammation, and Toll-like receptors in promoting atherosclerosis are well known [71]. Further studies are required to determine if circulating clusterin levels are indeed elevated in human MPS I patients, and if those levels correlate with severity of their cardiovascular disease.

Because GAGs are naturally present in healthy aortas [72], it will be important in the design of MPS cardiovascular disease therapeutics to determine if the arterial inflammation is caused solely by a quantitative increase of GAGs, or if the GAGs stored in MPS possess additional qualitative pro-inflammatory effects. The presence of storage-induced inflammation within MPS I canine arteries suggests several possible therapeutic strategies. Modified IDUA enzymes are being developed that have the potential to permeate deeper into the vascular parenchyma to alleviate GAG storage. An alternative strategy is the development of small molecule therapies that diffuse into arterial parenchyma and block the inflammatory response leading to MPS cardiovascular disease via inhibition of TLR4 signaling or pro-inflammatory cytokines. In fact, preclinical studies have been performed to investigate each of these modalities [73–76], and human clinical trials are in progress (European Union Clinical Trials Register # 2014-000350-11). Clusterin represents a promising biomarker to monitor efficacy of these therapeutic candidates in both preclinical and clinical studies for MPS I cardiovascular disease. The murine MPS I model system can be utilized to test efficacy of potential therapeutics targeted towards MPS cardiovascular disease.

**Supporting Information**

**S1 Appendix. Protocol for murine *Idua* genotyping.** This protocol was utilized for identifying the *Idua* genotype of the mice utilized in this study.

(PDF)
S1 Fig. Dendrogram and trait heatmap of the IDUA<sup>−/−</sup> and control canine aorta samples. The control samples cluster together and the IDUA<sup>−/−</sup> canine aorta samples cluster together. The same results are found if data from each tissue type is analyzed separately for up- and down-regulated genes, respectively. (TIF)

S2 Fig. Module trait-relationship table. Modules are designated on the left and the trait relationship is on the bottom (A for ascending aorta, C for carotid artery, and D for descending aorta). The table shows the correlation (top value) and the significance (bottom value) for each module-trait relationship. The turquoise and blue modules show the highest significance and thus were used for further analysis. (TIF)

S3 Fig. Identity of top perturbed genes within modules. In the turquoise and blue modules, further assessment identified the top genes based on fold-change and p-value using Student’s t-test. A. Volcano plot of the $-\log_{10}$ of the p-value vs. log<sub>2</sub> of fold change. B. Top up- and down-regulated genes in the module are represented in a bar graph with a log<sub>2</sub> scale. (TIF)

S4 Fig. TLR4 and pSTAT1 immunohistochemistry. Immunohistochemistry for demonstrates overexpression of TLR4 in canine MPS I aorta, but not in unaffected canine aorta. The presence of increased pSTAT1 in canine MPS I aorta compared to unaffected canine aorta is evidence for TLR4 activation. (TIF)

S5 Fig. Clusterin Western blotting. Scan of raw radiograph (Thermo Scientific CL-XPosure Film, ThermoFisher Scientific, Waltham, MA) exposure depicting enhanced chemiluminescence detection of canine aorta WB probed with anti-human clusterin alpha chain (EMD Millipore, Billerica, MA) and with anti-human smooth muscle alpha-actin (Dako North America, Inc., Carpinteria, CA). Hand written annotations refer to specific animal identifiers marking the lanes. Samples from descending or ascending aorta are grouped together and labeled as IDUA<sup>+/−</sup> (carrier) or IDUA<sup>−/−</sup> (MPS I). Handwritten markings indicate size and position of color coded protein ladder size markers (PageRuler Prestained Protein Ladder, Life Technologies, Grand Island, NY) traced by overlaying the film onto the transfer membrane. Clusterin is predicted to appear as a band between 35–39 kDa owing to differential glycosylation, while alpha smooth muscle actin is predicted to appear at approximately 42 kDa. (TIF)

S1 Table. Significantly upregulated genes within IDUA<sup>−/−</sup> canine aorta. The full list of mRNA probes showing significant (p-value ≤ 0.05) and greater than 2-fold level of upregulation in IDUA<sup>−/−</sup> canine arteries, compared to controls. Note that some genes are represented more than once due to existence of multiple mRNA probes per gene. (XLSX)

S2 Table. Significantly down-regulated genes within IDUA<sup>−/−</sup> canine aorta. The full list of mRNA probes showing significant (p-value ≤ 0.05) and greater than 2-fold level of down-regulation in IDUA<sup>−/−</sup> canine arteries, compared to controls. Again, some genes are represented more than once due to existence of multiple mRNA probes per gene. (XLSX)
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Author Contributions
Conceived and designed the experiments: OK MV PS PD JE RW. Performed the experiments: OK MV PG RW. Analyzed the data: OK MV PG NME PS PD JE RW. Contributed reagents/materials/analysis tools: OK PS PD JE RW. Wrote the paper: OK MV PG NME PS PD JE RW. Performed informatics, statistical analysis, Western Blotting densitometry: OK. Performed Western Blotting: MV.

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