Identification of novel proteins and mechanistic pathways associated with early-onset hypertension by deep proteomic mapping of resistance arteries

Resistance arteries are small blood vessels that create resistance to blood flow. In hypertension, resistance arteries undergo remodeling, affecting their ability to contract and relax appropriately. To date, no study has mapped the hypertensive-related proteomic changes in resistance arteries. Using a novel data-independent acquisition–mass spectrometry (DIA-MS) approach, we determined the proteomic changes in small mesenteric and renal arteries in pre- and early-onset hypertension from the spontaneously hypertensive rat (SHR) model, which represents human primary hypertension. Compared with normotensive controls, mesenteric arteries from 12-week-old SHRs had 286 proteins that were significantly up- or downregulated, whereas 52 proteins were identified as up- or downregulated in mesenteric arteries from 6-week-old SHRs. Of these proteins, 18 were also similarly regulated in SHR renal arteries. Our pathway analyses reveal several novel pathways in the pathogenesis of hypertension. Finally, using a matrisome database, we identified 38 altered extracellular-matrix-associated proteins, many of which have never previously been associated with hypertension. Taken together, this study reveals novel proteins and mechanisms that are associated with early-onset hypertension, thereby providing novel insights into disease progression.

Hypertension is the main risk factor for cardiovascular diseases and is a major global health burden, with increasing prevalence (1). Although many studies have investigated specific genes, proteins, and pathways that are altered in arteries from hypertensive animals and humans, there is no overview of the changes that occur in arteries during hypertension. As such, the pathophysiology of essential hypertension remains unclear. To advance research in the field of hypertension, we need a better overview of the changes occurring in arteries, which will promote new research ideas and potential therapeutic targets (2).

In hypertension, resistance arteries undergo eutrophic and/or hypertrophic remodeling, which contributes to increased peripheral resistance (3). In patients with essential hypertension and the spontaneously hypertensive rat (SHR), inward eutrophic remodeling predominates (3). Several mechanisms are proposed to influence vascular remodeling in hypertension (4), including apoptosis (5), expanded extracellular matrix (ECM) (6), vascular inflammation (7), and dysfunctional endothelium (8). In addition, several contractile and dilatory mechanisms are compromised in arteries from hypertensive animals and humans, which also contribute to the development and persistence of hypertension. These maladaptive changes in the vessel wall influence the development and cardiovascular complications of hypertension. Although proteins have been implicated in vascular remodeling in hypertension, these proteins do not work in isolation.

Mass spectrometry (MS) analysis has advanced rapidly over the past 2 decades and demonstrated clear advantages in mapping complex biological systems with high reproducibility (9, 10). Previously, MS analysis identified proteomic changes in the kidney (11), aortic smooth muscle (12), and left ventricular myocardium (13) in the SHR. The SHR develops elevated blood pressure (BP) between 7 and 15 weeks of age and mimics the central phenotypic changes observed in human essential hypertension, such as cardiac hypertrophy and vascular remodeling (14). To date, no study has mapped the proteomic changes in the resistance arteries of the SHR or in patients with essential hypertension.

The aim of this study was to investigate protein changes and mechanistic pathways in mesenteric resistance and renal arteries from the SHR, which is an ideal model for studying human essential hypertension without confounding lifestyle and environmental factors. Contrary to previous proteomic studies, we utilized next-generation data-independent analysis (DIA)-MS to achieve deep proteomic coverage of resistance arteries allowing us to identify novel proteins and map the pathophysiological mechanisms contributing to vascular remodeling and early-onset hypertension.

Results
Study overview and workflow
We investigated the protein composition of mesenteric artery samples from the SHR by label-free DIA quantification. Initially, we analyzed freshly isolated small mesenteric arteries from SHR and Wistar Kyoto (WKY) at both 6 and 12 weeks of...
age (Fig. 1A). BP in the SHR begins to increase at ~6 weeks of age, leading to a chronic elevated BP from ~12 weeks (14). Our study was designed to capture the critical changes that occur in the arterial wall during the early-onset of high BP; thus, these time points were selected to represent pre-hypertensive and early-onset hypertensive phenotypes and avoid confounding pathological changes associated with long-term chronic hypertension.

To achieve deep proteomic coverage, we generated a hybrid DIA library that was based on both high pH (HpH) reversed-phase peptide fractionated mesenteric artery samples and the strength of direct DIA to maximize the protein identification (15). Our hybrid library contained a total of 7450 proteins (73,378 peptides; 106,796 precursors; Fig. S1). Taking advantage of our hybrid DIA library, we identified a total of 4725 proteins in mesenteric artery samples from both SHR and WKY. Of these, 3956 proteins were consistently observed across all samples, suggesting high proteomic overlap and reproducibility between the mesenteric artery samples from SHRs and WKY controls (Fig. 1B).

Unbiased principal component analysis (PCA) revealed distinct clusters of mesenteric artery samples corresponding to age (6 and 12 weeks) along component 1 and phenotype (WKY and SHR) along component 2 (Fig. 1C). The separation of 6-week-old SHR and WKY control samples on component 2 was less compared with the 12-week-old samples, confirming...
higher proteomic similarity between the 6-week-old phenotypes. Using a volcano plot, we identified 17 regulated proteins, which accounted for the segregation of 6-week-old SHR and WKY control samples, including proteins such as Sept5, RGD:1562743, Flot1, Flot2, Gstt1, Naprt1, Lss, Acaa1a, Serpina3l, and Cecam1 (Fig. 1D). A total of 212 regulated proteins were identified in a volcano plot analysis when comparing 12-week-old SHR and WKY controls and supported the clear segregation seen in the PCA plot (Fig. 1, C and E). Almost all of the regulated proteins identified in the 6-week-old comparison (15/17) were shared across the two time point comparisons (Fig. 1, D and E).

Identification of 286 significantly regulated proteins in 12-week-old SHR mesenteric arteries compared with normotensive controls

The SHR is derived from the WKY (14) and inbred to perpetuate the hypertensive phenotype. Unfortunately, the litter-matched WKYs, from which the SHRs were identified, were not kept for inbreeding as a control. Subsequent attempts have been made to inbreed WKYS as a control, which is not ideal, thus the differences between the SHR and WKY controls might be due to strain differences and genetic drift rather than strain difference in BP (16). To control for this limiting factor in the animal model, we performed an additional DIA-MS analysis of mesenteric artery samples from six 13-week-old outbred Wistar Hannover rats. When comparing Hannover to WKY control, we observed a pronounced difference in the volcano plot (Fig. 2A). This difference was supported by a Student t test comparison identifying 1209 significantly regulated proteins between the normotensive Wistar strains (Fig. 2B).

The inclusion of SHR and WKY controls at two time points allowed analyses of relative proteomic differences in pre-hypertensive (6 weeks) and early-onset hypertensive (12 weeks) stages. Using Student t test analysis, we identified 52 and 360 significantly regulated proteins when comparing the mesenteric arteries of the SHRs to the WKY controls at 6 and 12 weeks of age, respectively (Fig. 2B; Tables S1 and S2). All candidate proteins contained between 2 and 307 unique peptides and were filtered by p and q values <0.05.

We compared the 360 significantly regulated proteins with those identified in the Hannover versus WKY control list and removed overlapping proteins that changed in the same direction. This resulted in the removal of 74 proteins from the SHR versus WKY list as these could be attributed to strain differences or genetic drift in the WKY rather than strain difference in BP. This conservative approach left us with 286 significantly regulated proteins (Fig. 2B; Tables S3 and S4). We focused on these proteome differences, thus the differences between the SHR and WKY controls might be due to strain differences and genetic drift rather than strain difference in BP. Using Student t test comparison, 1209 significantly regulated proteins were identified (Fig. 2A).

Fourteen different biological processes are associated with the protein changes in mesenteric arteries from the SHR

We ranked the top ten upregulated and downregulated candidate proteins based on the log2-transformed differences (Fig. 2C and Table 1). Examples included immunoglobulins, such as RGD:1562743 (Igkc), Igh-1a and Igg-2a, and thioredoxin-associated proteins, including Gstt3 and Tmx2. These proteins are highly associated with immune mechanisms, and the changes in expression levels suggested involvement of the immune system in SHRs.

The top ten regulated proteins had in average 16 unique peptides per protein, which suggests strong identification and gives confidence in the data (Table 1). To determine whether protein expression in any particular pathway was affected in the SHR, we performed pathway analysis on the entire list of significantly regulated proteins (=286) using ClueGO (17) (Fig. 2D and Table S5). The analysis identified 14 clusters of related Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms (Fig. 2D and Table 2). Each node was represented by 3 to 36 proteins and had an enrichment significance score between 4.85E-02 and 5.44E-10 using the Bonferroni step-down method (Table 2).

Mapping of the extracellular-matrix-associated protein changes in the SHR mesenteric arteries

Arteries from the SHR undergo vascular remodeling with thickening of the wall accompanied by reduction in lumen diameter (3). Using Sirius red staining, we confirmed the presence of vascular remodeling in 12-week-old SHRs compared with WKY controls (Fig. 3, A and B). In a blinded image analysis, we observed significant increases in media-to-lumen ratio in mesenteric arteries from the SHR compared with WKY controls, validating the presence of hypertrophic remodeling (Fig. 3C; Student t test comparison, p < 0.0001, t = 7.329, df = 37). Remodeling of the ECM has been associated with vascular remodeling (6). To explore this, we took advantage of our list of significantly regulated proteins and enriched for cellular components in an additional pathway analysis using ClueGO, which identified the collagen-containing extracellular matrix pathway (Fig. 3D and Table S6). To further elucidate this association, we compared our total protein list (before removal of the outbred Hannover-associated proteins) to a “matrisome” gene list containing ECM and ECM-associated proteins (18, 19) and identified a total of 228 proteins that were associated with the matrisome. A new unbiased PCA plot, based on the matrisome-associated proteins only, revealed an almost identical clustering of samples as observed in Figure 1C that included all proteins (Figs. 1C and 3E). Notably, collagen Col5a3 and the two ECM glycoproteins, Agrn and Mfge8, showed specific enrichment toward WKY controls and SHRs, respectively (Fig. 3F). Only six proteins (Mfge8, Plod1, F9, Vwf, P4ha2, and Col5a3) were significantly regulated in the 6-week comparison (Fig. 3G), whereas 38 proteins were regulated in the 12-week comparison (Fig. 3H). Mfge8 was identified as the most upregulated ECM protein in both 6- and 12-week-old rats (p = 5.00E-04 and 2.00E-04, respectively). Vwf was the most downregulated protein at 6 weeks (p = 3.60E-04, Fig. 3G) while Serpina6 was the most downregulated protein at 12 weeks (p = 8.28E-05, Fig. 3H). Although it did not reach statistical significance in the 12-week comparison, Col5a3 was the most downregulated
protein identified with a difference of −2.14 (p = 0.073; Fig. 3H).

Unsupervised hierarchical clustering of significantly regulated ECM-associated proteins revealed three major groups (Fig. 3I). Cluster 1 was mainly upregulated in 12-week-old SHRs and contained mostly ECM glycoproteins (ε, n = 7/8; Fig. 3J). Cluster 2 was particularly upregulated in 12-week-old WKY controls and contained mostly ECM regulators (θ, n = 11/15; Fig. 3J). Cluster 3 contained a mixture of several ECM types and was generally upregulated in both 6- and 12-week-old WKY controls (Fig. 3J).

**Maintained protein changes across vascular beds of the 12-week-old SHR: Analysis of the renal arteries**

To determine which proteomic changes of early-onset hypertension that were identified in the mesenteric arteries and
were maintained in a different vascular bed, we analyzed renal arteries from the SHR. Using the same sample preparation and DIA-MS setup, we consistently observed 3727 proteins across renal artery samples that were shared between SHRs and WKY. Furthermore, the top ten regulated proteins detected in the 12-week-old WKY and SHR renal arteries. Of these, 57 were significantly regulated protein list identified when comparing the proteomic profile of 12-week-old mesenteric arteries from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). The number of unique peptides (precursors) identified per protein is included.

To validate the MS data, we selected a protein from the top up- and downregulated proteins from 12-week-old mesenteric arteries from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). The number of unique peptides (precursors) identified per protein is included.

Critical changes in protein expression across vascular beds

Our experimental setup enabled us to investigate the overlap of significantly regulated proteins between 6- and 12-week-old mesenteric arteries from the SHR compared with WKY control. We found that 30 proteins were shared between both stages (Fig. 6A), suggesting a central involvement in early BP regulation in the SHR. To clarify the potential importance of these in the vascular network in general, we compared the 30 significantly regulated proteins identified in our renal artery analysis. This left us with 18 proteins (Fig. 6A and Table 4) that were changed across two different vascular beds.
Proteome of arteries in hypertension

Table 2
Pathway analysis of proteins associated with hypertension phenotype

| Pathway                                | Ontology source | Corrected p-value | Upregulated | Downregulated |
|----------------------------------------|-----------------|-------------------|-------------|--------------|
| Acute inflammatory response            | GO Biological Process | 3.4E-03           | Park7       | Ahsg, Apoa2, F2, Icam1, Itih4, Kng1, Kng2, Pla2g4a, Serpinb9 |
| Cellular modified amino acid metabolic process | GO Biological Process | 1.13E-04          | Aldh7a1, Clk, Gstt1, Gstt3, Park7, Tm1he | Aldh9a1, Cpq, Crot, Gstm5, Idh1, Kyat1, Por, Slc27a1 |
| Extracellular exosome                   | GO Cellular Component | 2.70E-02          | Hspd1, Park7, Pdcd6, Sdcbp | Alb, Icam1, Sriti |
| Generation of precursor metabolites and energy | GO Biological Process | 5.07E-04          | Agl, Aldoc, Atpp5o, Coq9 | Adh1, Apoc3, Crot, Cyb5a, Eno2, Idh1, Pgam2, Pgd, Por, Tkt |
| Glycolysis/Gluconeogenesis              | KEGG            | 9.96E-03          | Acs1, Aldh7a1, Aldoc | Adh1, Alldh9a1, Eno2, Pgam2 |
| Hypertrophic cardiomyopathy            | KEGG            | 4.28E-02          | Atpp2a2, Itg7, Prkag2, Ryr2 | Acs1, Uba3 |
| Ligase activity, forming carbon-sulfur bonds | GO Molecular Function | 3.00E-02          | Acs1, Aldoc, Mapk14, Park7, Prkag2 | Acs5, Slc27a1, Slc27a4 |
| Monocarboxylic acid metabolic process  | GO Biological Process | 1.76E-05          | Acs1, Aldoc, Mapk14, Park7, Prkag2 | Acs5, Acs6, Adh1, Agt, Apoa4, Apoc1, Apoc3, Ces1d, Crot, Eno2, Epfx1, Hyl, Idh1, Kyat1, Pgam2, Pgd, Pla2g4a, Por, Sgpl1, Slc27a1, Slc27a4, Tectr |
| Negative regulation of hydrolyase activity | GO Biological Process | 5.44E-10          | Cnt3, Crim1, Ddx3x, Farp1, Fkbpl1a, Gas6, Park7, Pcsk11, Pppr14a, Rock1 | Acs5, Acs6, Adh1, Agt, Apoa4, Apoc1, Apoc3, Ces1d, Crot, Eno2, Epfx1, Hyl, Idh1, Kyat1, Pgam2, Pgd, Pla2g4a, Por, Sgpl1, Slc27a1, Slc27a4, Tectr |
| Negative regulation of response to wounding | GO Biological Process | 8.85E-05          | Cask, Neo1, Phld2 | Cers2, Cbp2, F2, Kng1, Kng2, Proc, Thbd, Coll3a1, F2, Pla2g4a |
| Platelet activation                    | KEGG            | 1.36E-02          | Gucy1a2, Ipir1, Mapk14, Mylk, Ppp1cc, Rock1 | Adh1, Ahsb, Apoa2, Apoa4, Cers2, Cbp2, Cyp2, Cyp, Gm, Sgca |
| Regeneration                           | GO Biological Process | 4.72E-04          | Aldoc, Gas6, Ipir1, Lamb2, Mapk14, Mstn1, Neo1, Ptgfrn | Acs5, Coasy, Crot, Eno2, Mcee, Pdeled, Pgam2, Tkt |
| Ribose phosphate metabolic process     | GO Biological Process | 1.67E-02          | Acs1, Aldoc, Atpp5o, Cpts1, Cycl1, Gucy1a2, Prkag2, Upt | Aac1a1, Adh1, Ces1d, Crot, Ddhah1, Eno2, Gpd2, Kyat1, Pgd, Slc27a4 |
| Small molecule catabolic process        | GO Biological Process | 4.28E-02          | Esd, Gnpda2, Oxc1t1, Park7 | Acs5, Acs6, Adh1, Agt, Apoa4, Apoc1, Apoc3, Ces1d, Crot, Eno2, Epfx1, Hyl, Idh1, Kyat1, Pgam2, Pgd, Pla2g4a, Por, Sgpl1, Slc27a1, Slc27a4 |

ClueGO-enrichment analysis of significantly regulated proteins identified in mesenteric arteries from comparing 12-week-old Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR). The annotations represent related Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) terms enriched as nodes. Each node is labeled with the term having the highest significance. p-value correction was calculated by Bonferroni step-down method.

Discussion

This study provides novel insight into proteins that are changed in small mesenteric resistance and renal arteries in SHR during the development of early-onset hypertension, most of which have never been associated with hypertension previously. Our experimental design allowed us to characterize protein changes before and after the development of hypertension in the SHR. In addition, by investigating protein changes in the renal arteries, we reveal 18 candidate proteins that change critically in different vascular beds. Only four out of the 18 proteins have been associated with hypertension (20–23); however, the pathophysiological role of all proteins is undetermined. To achieve the comprehensive proteomic depth, we used a next-generation DIA-MS approach with a hybrid library combining HpH reversed-phase peptide fractionated DDA data and power of direct DIA. To our knowledge, we are the first to establish a rat mesenteric resistance artery-based library, which is ideal for a discovery-based proteomic investigation in rat arteries. The hybrid library has been made publicly available allowing others to access the data, from which new hypotheses can be generated, thereby advancing research in the hypertension field.

Several genetic differences exist between the SHR and WKY control strain because of selective inbreeding (24). Despite these differences, the WKY strain is considered the closest control available. To compensate for inbreeding differences, we included a comparison to an outbred Wistar Hannover strain. By using this additional strain, we could identify proteins that were likely to be due to genetic drift and changed beds and thus could contribute to a common regulatory mechanism of early-onset hypertension. Notably, the 18 proteins were identified after filtering for several criteria that limited false-positive discovery significantly, including: (1) more than two unique peptides per protein, (2) q-value (FDR) cutoff >0.05, significance cutoff by adjusted p-value >0.05, (3) removal of proteins that were potentially affected by selective inbreeding, (4) significance across two arterial beds. To elucidate the biological relevance, we performed an unsupervised hierarchical clustering analysis of the 18 proteins that revealed regulation of five clusters of proteins (Fig. 6B). Using literature mining on the clustered proteins, we identified associations to protease inhibition, intracellular Ca2+ concentration, immunoglobulins, ECM, lipid metabolism, glutathione metabolism, remodeling and membrane excitability, reactive oxygen species (ROS), microtubules, glycosylation, and collagen synthesis and degradation.
protein expression in the WKY, rather than strain difference in BP, and remove these proteins from our analysis. Although not within the scope of this study, the number of significantly regulated proteins between the WKY and Hannover was surprisingly high (=1209), suggesting a high proteomic difference between the two control strains. This should be taken into consideration when groups compare conflicting data obtained from similar experiments in the different strains. Nevertheless, only 74 of the regulated proteins between the WKY and Hannover matched those in the SHR versus WKY list, which left 286 significantly regulated proteins that were associated with a strain difference in BP in the SHR mesenteric arteries. This list represents the proteins that show the highest degree of expression changes from the SHR mesenteric arteries, which allowed us to investigate novel mechanistic pathways involved in the early stages of hypertension. Our enrichment analysis of the 286 regulated proteins revealed monocarboxylic acid metabolic process and negative regulation of hydrolase activity as the predominant pathways associated with early-onset hypertension. We observed that the monocarboxylic acid metabolic process cluster was particularly associated with lipid metabolism processes such as regulation of lipid metabolic process, fatty acid metabolic process, and regulation of lipase activity. The regulated proteins in this pathway suggest a
change in the handling of lipids in the vascular wall of the
SHRs. For instance, decreased APOA4, APOC1, and APOC3
expression would result in reduced lipid removal from the
vascular wall (25) and inhibition of lipoprotein lipase activity
(26), potentially promoting lipid accumulation, which might be
associated with hypertension (27). Changes in the expression
of proteins involved in signaling pathways can be dif-
cult to interpret in a proteomic study such as this. The activation
states of such proteins are not reflected by their overall level of
expression, thus it can be difficult to determine whether the
activity of a pathway is truly up- or downregulated. Therefore,
it is important that future studies investigate the pathophys-
ological contribution of specific pathways detected in this
study, which are potentially involved in the development of
hypertension.

ECM proteins play a critical role in vascular stability and cell
behavior, with dysfunction in this system linked to the path-
ogenesis of hypertension (6); however, a complete overview of
the ECM-associated remodeling in hypertension has never
been established. Thus, with the extensive proteomic coverage

Figure 4. Pathway analysis of proteins associated with early-onset hypertension in renal arteries from the SHR. A, stacked bar representation of protein groups identified by data-independent acquisition mass spectrometry (DIA MS) across renal artery samples (blue = complete identifications, orange = shared in 50% of runs, red = sparse identifications). B, Venn diagram showing total number of exclusive and shared protein groups identified in mesenteric and renal arteries (orange and blue circle, respectively). C, volcano plot comparing protein abundance in 12-week-old renal artery samples from spontaneous hypertensive rat (SHR, n = 7) and Wistar Kyoto (WKY, n = 7) control. D, representation of top ten up- (left, red) and top ten downregulated (right, blue) proteins when comparing 12-week-old SHR and WKY control. E, ClueGO-enriched network of significantly regulated proteins identified when comparing 12-week-old SHRs and WKY controls with t test analysis. The protein list was enriched against the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.
achieved in this study, we created a map of ECM changes in arteries from the SHR. Our data revealed 38 ECM-related proteins that were regulated in the SHR at 12 weeks of age, most of which have not been linked to hypertension previously. Interestingly, our data indicated that the quantified ECM proteins could be grouped into three distinct clusters based on (1) ECM glycoproteins, (2) ECM regulators, and (3) a mixture of ECM proteins. These clusters could distinguish the SHR from the WKY controls, supporting a phenotypical difference. Notably, some of the identified ECM glycoproteins in our study have been linked to vascular remodeling and stiffness previously. For example, elevated expression of MFGE8 was positively correlated with aortic stiffness in chronic kidney disease patients (28), and increased GAS6 levels in serum have been observed to correlate with elevated BP and age-related vascular remodeling in mice (29). In addition, increased plasma levels of SPARCL1 have been found in patients with maladaptive right ventricular function from pulmonary hypertension (30). We identified similar regulations of Mfge8, Gas6, and Sparcl1 in our study, supporting a pathological relevance of the identified ECM glycoproteins. The ECM regulators were highly abundant in 12-week-old WKY controls while the expression levels were lower in the three other groups. Several of these proteins, such as inter-alpha-trypsin inhibitor heavy chain (Itih2–4) and the serpins, improve ECM stability (31). Although their role in ECM stability in the vascular wall is yet to be determined, our data suggest that downregulation of these proteins in hypertension could destabilize the arterial ECM. Notably, the studies referred to here (28–30) investigated individual proteins and their pathological association to remodeling. While our MS method detected these proteins, our approach also identified many other coclustering proteins that were regulated and thus a more complex interplay between ECM proteins.

Table 3
Top ten regulated proteins in renal arteries

| Protein group(s) | Genes | Protein description | Log2 difference | p-value Unique peptides |
|------------------|-------|---------------------|-----------------|------------------------|
| Top ten upregulated proteins | P01836 | RGD:1562743 Ig kappa chain C region, A allele | 4.64 | 5.96E-08 7 |
| D3Z8I7 | Gstt3 | GST N-terminal domain-containing protein | 2.33 | 3.84E-11 9 |
| P80254 | Ddt | D-dopachrome decarboxylase | 1.73 | 5.47E-08 11 |
| A0A0G2K7B6 | Dysf | Dysferlin | 1.26 | 1.33E-03 68 |
| Q4V8C2 | Zw10 | Centromere/kinetochore protein zw10 homolog | 1.26 | 1.43E-03 10 |
| Q4Q8R0 | Mrps25 | 28S ribosomal protein S25, mitochondrial | 1.19 | 4.41E-04 6 |
| F1LLV6;M0R7R1;Q63010 | Ces1f;Ces1f- | Isoform of P10959, Carboxylic ester hydrolase; Isoform of P10959, Carboxylic ester hydrolase; Liver carboxylesterase B-1 | 1.16 | 1.21E-04 8 |
| Q9Z1E1 | Flot1 | Flotillin-1 | 1.16 | 3.57E-09 40 |
| F1M455;F1M816 | Spen | Spen family transcriptional repressor; Isoform of F1M455, RCG30673 | 1.02 | 3.70E-03 2 |
| Q9Z2S9 | Flot2 | Flotillin-2 | 1.00 | 3.03E-08 44 |
| Top ten downregulated proteins | Q63910 | Hba-a3 | −6.06 | 1.64E-04 17 |
| G3V6F6 | Fmo2 | Isoform of Q6IRI9, Dimethylaniline monooxygenase [N-oxide-forming] | −3.06 | 1.44E-04 29 |
| P20767 | ENSRNOG00000050000 | Ig lambda-2 chain C region | −2.11 | 3.17E-06 7 |
| P05544 | Serpina3l | Serine protease inhibitor A3L | −2.04 | 3.67E-07 51 |
| P20761 | Igj-1a | Ig gamma-2B chain C region | −1.96 | 4.50E-06 24 |
| P18589;Q499Q3 | Mx2 | Interferon-induced GTP-binding protein Mx2; Isoform of P18589, Interferon-induced GTP-binding protein Mx2 | −1.93 | 1.71E-03 17 |
| A0A0G2K477 | ENSRNOG00000034190 | Isoform of F1LPW0, Immunoglobulin heavy constant mu | −1.93 | 2.00E-06 16 |
| A0A0G2K980;M0RBD5 | ENSRNOG00000055375 | Ig-like domain-containing protein; Isoform of A0A0G2K980, Ig-like domain-containing protein | −1.92 | 1.15E-05 2 |
| A0A0G2JVP4;F1LM30;F1LPW0 | ENSRNOG00000034190 | Isoform of F1LPW0, Immunoglobulin heavy constant mu; Isoform of F1LPW0, Immunoglobulin heavy constant mu; Immunoglobulin heavy constant mu | −1.90 | 1.28E-07 23 |
| D3ZF97 | Erlec1 | Endoplasmic reticulum lectin 1 | −1.89 | 7.62E-04 4 |

Description of the top ten up- and downregulated proteins identified when comparing the proteomic profile of 12-week-old renal arteries from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). The number of unique peptides (precursors) identified per protein is included to support the identification.
highlights the advantage of using a discovery-based DIA-MS approach to unravel the complex compositional and dynamic changes that occur during arterial wall remodeling.

Despite a large overlap of detected proteins between the mesenteric and renal arteries (=4135), the number of shared significantly regulated proteins was limited between the two artery types (=53). Other than these being different vascular beds, the renal arteries were also conduit arteries compared with resistance mesenteric arteries, which is likely to contribute to this proteomic difference. Despite the small overlap, one biological process (cellular modified amino acid metabolic process) was shared between the two pathway analyses of renal and mesenteric arteries. When comparing the associated proteins from both analyses, we observed that the majority (Eef1g, Idh1, Gsto1, Gstt1, and Gstt3) were associated with glutathione metabolism, a child term of the cellular modified amino acid metabolic process. It has been proposed that glutathione can serve as an intracellular thiol-disulfide redox buffer that can protect against oxidative stress due to its oxidizability (32). Increased levels of red blood cells containing glutathione in its oxidized state have been reported in untreated hypertensive patients (33). Furthermore, increased levels of superoxide $O_2^{-}$ have been observed in spontaneously hypertensive stroke-prone rats (34), linking glutathione metabolism to hypertension. The glutathione S-transferases (GSTs), such as Gsto1, Gstt1, and Gstt3, catalyze glutathione-dependent reactions leading to conjugation and detoxification of ROS in vascular smooth muscle cells (VSMCs) (35). As such, upregulation of Gsto1, Gstt1, and Gstt3 in the SHR could occur as a counteracting mechanism to cope with increased...
levels of vascular oxidative stress. Interestingly, another variant of GSTs (Gstm5) was observed to be reduced in renal specimens from hypertensive patients using genome-wide microarray expression profiling (36). We similarly observed a reduction of Gstm5 in mesenteric and renal arteries (p = 0.003 and 0.669, respectively). Although this supports our findings, it also implicates an inverse relationship between the expression levels of GST variants in the SHR (e.g., upregulation of Gstt1/3 variants and downregulation of Gstm5).

Importantly, our experimental setup allowed us to reveal 18 proteins that were regulated significantly in pre- and early-onset hypertension in the mesenteric artery, which were also altered in the renal artery. From these 18 proteins, we identified five pathway clusters, which are likely to be driving the hypertensive phenotype in different vascular beds. Cluster 1 included four proteins Serpin3l, Igg-2a, ENSRNOG00000049829, and Acyp2 that associated with regulation of protease inhibition, intracellular Ca²⁺ concentrations, and immunoglobulins. Serpin3l is a serine protease inhibitor that also was included in the pathway analysis of 12-week-old mesenteric arteries (negative regulation of hydrolase activity; alias LOC299282). The inhibitor was among the most downregulated proteins in both artery types in the SHRs. These protease inhibitors might have a protective role against ECM remodeling, which is likely to be lost when downregulated. Interestingly, the SR calcium pump regulator Acyp2 enhances SERCA2a activity (37, 38), thereby regulating transport of cytosolic Ca²⁺ into the SR. The concentration of cytosolic Ca²⁺ in VSMCs regulates vascular tone and remodeling, which is critically associated with hypertension (39–42). The expression level of Acyp2 was downregulated in 6- and 12-week-old SHRs, which could lead to changes in Ca²⁺ homeostasis. Interestingly, our data showed increased expression levels of Ryr2 in mesenteric arteries from the SHRs. The Ryr2 channel is also involved in releasing Ca²⁺ from the SR, supporting changes in intracellular Ca²⁺ regulation between the SHRs and WKY controls. These data suggest that a suppression of SERCA2a and increased level of Ryr2 reduce the Ca²⁺ levels in SR, increase cytosolic Ca²⁺, thereby affecting the contractile state of VSMCs.

Cluster 2 included Enpp3, Lss, Acaa1a, Basp1, and Basp1 isoform, which associated with ECM and lipid metabolism. Enpp3 is a hydrolizing glycoprotein involved in regulating extracellular nucleotides. Enpp3 mRNA expression was downregulated in VSMCs when exposed to angiotensin II (43). Although...
Proteome of arteries in hypertension

### Table 4
**Detected driver proteins**

| Protein group(s) | Genes | Protein description | Molecular weight | Unique peptides |
|------------------|-------|---------------------|------------------|----------------|
| A0A0A0MY07;Q9R085 | Usp15 | Isoform of Q9R085, Ubiquitin carboxyl-terminal hydrolase/Ubiquitin carboxyl-terminal hydrolase 15 | 109240.56;109254.59 | 18 |
| A0A0G2JY14;D3ZGT6 | P4ha2 | Isoform of D3ZGT6, Procollagen-proline 4-dioxigenase/Procollagen-proline 4-dioxigenase | 60635.84;60867.06 | 28 |
| A0A0G2K1L8 | Basp1 | Isoform of Q05175, Brain acid soluble protein 1 | 21719.25 | 2 |
| D4A1G1;P35745 | Acyp2 | Isoform of P35745, Acylphosphatase-Acylphosphatase-2 | 13644.71;10863.3 | 6 |
| F1LP76;Q8VHU4 | Elp1 | Isoform of Q8VHU4, Elongator complex protein 1/Elongator complex protein 1 | 149198.57;149170.64 | 22 |
| F1LTN6 | ENSRNOG00000049829 | Uncharacterized protein | 24914.85 | 20 |
| P05544 | Serpina3a | Serine protease inhibitor A3L | 46727.17 | 46 |
| P20760 | Igf-2a | Ig gamma-2A chain C region | 35185.86 | 25 |
| P21775 | Acaa1a | 3-ketoacyl-CoA thiolase A, peroxisomal | 43833.22 | 14 |
| P48450 | Lss | Lanosterol synthase | 83300.54 | 16 |
| P97675 | Enpp3 | Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | 99071.63 | 40 |
| Q01579 | Gstt1 | Glutathione S-transferase theta-1 | 27468.29 | 12 |
| Q05175 | Basp1 | Brain acid soluble protein 1 | 21790.27 | 20 |
| Q566E5 | Poglut3 | Protein O-glucosyltransferase 3 | 58701.34 | 21 |
| Q6DGG0 | Ppid | Peptidyl-prolyl 4-dioxygenase;Procollagen-proline 4-dioxygenase | 40765.68 | 20 |
| Q7TQ94;Q7TQ94-2 | Nit1 | Deaminated glutathione amidase;Isoform of Q7TQ94, Isoform 2 of Deaminated glutathione amidase | 36093.54;36093.54-2 | 23 |
| Q9Z1E1 | Flot1 | Flotillin-1 | 47499.4 | 54 |
| Q9Z2S9 | Flot2 | Flotillin-2 | 47038.04 | 55 |

Description of the 18 proteins that are shared across two vascular beds (mesenteric and renal arteries) when comparing spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto (WKY) rats. The number of unique peptides (precursors) identified per protein is based on the mesenteric artery analysis.

Angiotensin II is not the main driver of increased BP in the SHR (44), the model has increased renin and angiotensin levels in serum (45), suggesting pathological similarities to the angiotensin II-induced model. Our data showed reduced Enpp3 expression in SHRs compared with WKY controls, which is likely due to increased angiotensin II levels in the SHR. Lss and Acaa1a are associated with lipid metabolism by regulating cholesterol and fatty acid synthesis. There is a strong link between dyslipidemia and hypertension, supporting the regulation of these proteins (46).

Cluster 3 included Gstt1, Nit1, Flot1, and Flot2 and associated with glutathione metabolism, remodeling, and membrane excitability. Both Gstt1 and Nit1 are linked to glutathione metabolism and were upregulated in both 6- and 12-week-old SHRs, supporting the glutathione metabolism pathway association that was observed in renal arteries. The flotillins, Flot1 and Flot2, are membrane-associated proteins that are involved in cell-matrix adhesion, endocytosis, and can assemble lipid rafts or microdomains that function as signaling platforms (47, 48). Our data showed that Flot1 and Flot2 were among the most upregulated proteins in SHRs, and we validated Flot1 changes by Western blot and IHC analysis, which supported the detection. Notably, expression levels of Flotillins-2 were significantly increased in cardiac intercalated disk fractions from both Dilated Cardiomyopathy (DCM) and Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) patients (49). Both diseases are associated with cardiomyocyte remodeling, and links increased flotillin-2 to a remodeling phenotype. Furthermore, decreased expression of the cardiac sodium channel Nav1.5 accompanied with impaired cardiac conductance was reported in Flotillin-1/2 knockdown mice compared with control (50). Flotillins have, to our knowledge, not been studied in relation to hypertension and vascular remodeling, but our study suggests a critical role of flotillins in the pathophysiology of hypertension.

Cluster 4 contained three proteins including Ppid, Ikbkap, and Poglut3 that associated with changes in ROS, microtubules, and glycosylation. Ppip or Cyclophilin D regulates the mitochondrial permeability transition pore and is a regulator of mitochondrial ROS generation (51). Angiotensin II-induced hypertension increased Ppip-associated ROS production in mice (52). Conversely, angiotensin II-induced hypertension was attenuated when using Ppip-depleted mice, demonstrating a link between ROS production and Ppip in the hypertensive mice (52). Our data also showed an increased expression of Ppip in SHRs, which is likely due to elevated angiotensin levels in the SHRs. Ikbkap (or Elp1) is a scaffold protein that can promote α-tubulin acetylation, thereby regulating microtubule network remodeling and dynamics (53, 54). We have previously shown that the microtubule network is an important trafficking pathway for certain proteins (55, 56). Although, little is known about the effect of hypertension on the
microtubule network, one study reported that angiotensin II-treatment enhanced deacetylation and disassembly of tubulin in endothelial cells (57), questioning whether exposure to a high BP causes changes to the microtubule stability. Our data showed increased Ikbkap expression in SHRs compared with controls and could support a counteracting mechanism by Ikbkap in the SHRs. The Poglut3 enzyme facilitates O-glycosylation on epidermal growth factor (EGF)-like repeats on a variety of proteins during secretion (58). We found that Poglut3 expression increased in SHRs compared with WKY control. This is in alignment with our observation of several ECM-associated glycoproteins that increased in SHRs. However, changes in Poglut3 expression can affect multiple pathways, and therefore Poglut3 might contribute to the pathophysiology of hypertension in several ways. For example, glycosylation is also required for Notch processing and trafficking (58), and changes in Notch3 signaling have been proposed to play a critical role in VSMC differentiation and the pathogenesis of pulmonary arterial hypertension (59).

Cluster 5 included two proteins P4ha2 and Usp15 that associated with collagen synthesis and degradation. P4ha2 can facilitate the triple helix formation of collagen and regulates the hypoxia-inducible transcription factor HIFα (HIF-1α) by catalyzing 4-hydroxyproline residues (60). HIF-1α can modulate ECM formation via P4ha2 or other regulators such as Sox9 (61). We found decreased P4ha2 expression in SHRs that is in alignment with our matrisome profiling data, which also showed decreased expression of collagens, such as Col3a1 and Col5a3 in the SHRs compared with WKY control. The enzyme Usp15 catalyzes deubiquitination and regulates the ubiquitin-proteasome system by stabilizing monomers of ubiquitin on proteins. Inhibition of USP15 decreased collagen expression, including Col3a1, in TGF-β-stimulated fibroblast cell cultures, suggesting that USP15 can regulate collagen expression (62). Notably, our data showed decreased expression of both Usp15 and Col3a1 in SHRs, and the regulatory effect of Usp15 might therefore explain the downregulation of Col3a1.

Taken together, we identified associations to protease inhibition, intracellular Ca2+ concentrations, immunoglobulins, ECM, lipid metabolism, glutathione metabolism, remodeling and membrane excitability, ROS, microtubules, glycosylation, and collagen synthesis and degradation. Although elements of these pathways have been linked to hypertension previously (4, 63), this study is the first to show collective proteins that are linked with each pathway, which are dysregulated in the prehypertensive stage of the disease and not elicited by pressure changes. This study presents vast amounts of data implicating several novel proteins in the pathogenesis of hypertension, as well as highlighting these 18 proteins and their associate pathways to provide novel insight into the disease, which, with further investigation, have the potential to become innovative therapeutic targets.

This is an explorative study, which is not hypothesis-driven. As such, we have not been able to investigate the physiological impact of the novel proteins we have identified in the pathogenesis of hypertension. However, the enrichment analysis provides mechanistic insights into the pathways involved in the development of hypertension, thereby creating a plethora of new hypotheses, which will be tested in future studies and substantially advance the field. Furthermore, our proteomic analysis did not differentiate between cell types found in the vascular wall. Although it would be advantageous to have cell-type specific changes in protein expression, the process of isolating cells can influence the protein expression profiles and limits the ability to analyze changes in ECM-associated proteins. As such, we see it as an advantage that the study is based on intact, freshly isolated arteries. Our proteomic analysis provides a firm foundation for hypotheses on cell-specific modifications of the vascular wall in hypertension.

In summary, this study has unraveled the deep proteomic complexity of mesenteric resistance arteries in SHRs and WKY controls. We identified changes in several ECM proteins providing novel insight into the vascular remodeling process observed in the SHRs. Additionally, our data reveal 18 proteins driving the prehypertensive phase as well as early-onset hypertension. Our pathway analysis of these driver proteins demonstrates an involvement of multiple novel proteins and pathways that have not been associated with hypertension previously. Together, these data will generate new hypotheses and advance the field of hypertension.

**Experimental procedures**

**Experimental animals**

The animal experiments were approved by local Animal Care and Use Committees (institutional approval numbers P20-457 and P21-117). Experiments were performed in accordance with the directives of the Danish National Committee on Animal Research Ethics, and Danish legislation on experimental animals. In accordance with the methods of killing animals described in annex IV of the EU Directive 2010/63EU, rats were made unconscious by a single, percussive blow to the head. Immediately after the onset of unconsciousness, cervical dislocation euthanized the rats. Three cohorts of male SHRs (SHR/KyoRj) (14), WKYs (WKY/KyoRj), or Hannover rats (Janvier) at 6 weeks, 12 weeks (SHRs and WKYs), and 13 weeks (Hannover) of age were group housed and supplied with ad libitum water and food access (n = 4 (6-week groups), 7 (12-week groups), and 6 (Hannover group), respectively). Clean cages were provided once a week, and rats were kept on a 12 h/12 h light/dark cycle.

**Measurement of blood pressure**

To avoid confounding effects of anesthesia in the proteomic analysis, three rats from each group were sampled in order to determine the BP. These rats were not included in the proteomic analysis, but represent the BP of the population of rats used in the study. Increased mean BP over a stable 5 min period was confirmed in SHRs compared with WKY (Mean BP ± standard deviation: SHR = 140.975 ± 29.468, WKY = 79.577 ± 13.393, n = 3 in each group). The BP was measured as described previously (64). In brief, rats were anesthetized with 5% isoflurane (35% oxygen and 65% nitrogen), intubated, and connected to a respirator (=65 breaths/min; tidal volume 8 ml/kg).
Proteome of arteries in hypertension

The left carotid artery was cannulated with a catheter connected to a pressure transducer (Statham P23-dB) for continuous monitoring of the BP. A heating plate was used to maintain the body temperature of the rats at 37 °C. After the experimental protocol, the rats were euthanized using cervical dislocation.

Dissection of arteries

After the rats were euthanized, the intestines containing the mesenteric vascular bed and the main branch of the renal artery connecting the thoracic aorta to the kidney were excised and incubated in ice-cold physiological salt solution (PSS: 120 mM NaCl, 2.8 mM KCl, 1.5 mM CaCl₂, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 2.5 mM MgSO₄, 0.03 mM EDTA, 5.6 mM D-glucose). Small mesenteric and renal arteries were dissected, collected in 1 ml Lobind centrifuge tubes (Eppendorf), snap frozen in liquid nitrogen, and stored at −80 °C. Small sections of mesenteric resistance artery (0.5−1 cm in length) were embedded in Tissue-Tek OCT (Sakura) for sectioning and staining.

Protein isolation and quantification

Snap-frozen arteries were homogenized in 200 μl of ice-cold lysis buffer (50 mM Tris pH 8.5, 5 mM EDTA pH 8.0, 150 mM NaCl, 10 mM KCl, 1% NP-40 and 1× complete protease inhibitor cocktail (Roche)) by three rounds of chopping the tissue using dissection scissors and a handheld homogenizer. Homogenates were centrifuged at 11,000g for 10 min at 4 °C to obtain the supernatant. Protein quantification of the tissue extracts was determined by bicinchoninic acid assay (BCA) (Thermo Scientific).

Tissue sectioning, staining, and imaging

Small mesenteric resistance arteries were sectioned in a cryostat microtome (Leica CM3050 S). Sections were cut at 10 μm thickness and attached to Superfrost Plus glass slides (VWR) and stored at −80 °C. Tissue sections for bright field imaging were stained with a Sirius red staining protocol. In brief, tissue sections were adjusted to room temperature (RT) and fixed in Bouin’s solution (Sigma) overnight (O/N). Sections were rinsed in Milli-Q water for 20 min, stained in Weigert’s solution for 10 min (filtered Weigert HTX A solution and Weigert HTX B solution, Histolab), and rinsed in Milli-Q water for 5 min. Sections were stained in filtered Picro-sirus red solution for 15 min (Histolab), dehydrated in 99% EtOH, washed in xylene (Sigma), and allowed to air-dry before mounting in pertex (Histolab).

Tissue sections for fluorescent imaging were fixed in 4% PFA/1×PBS (15 min), washed in 1×PBS, blocked in blocking buffer (5% normal swine serum (Jackson ImmunoResearch), 1% bovine serum albumin (BSA, Sigma), 0.1% TritonX-100 (Sigma) in 1×PBS), and stained with commercial anti-FLOT1 (HPA001393, 1:500) and anti-FMO2 (HPA028261, 1:300) from (Sigma) diluted in 1% BSA, 0.1% TritonX-100 in 1×PBS for 1 h at RT. Washes in a washing buffer (0.25% BSA, 0.1% TritonX-100 in 1×PBS) were used between and after antibody staining. Hoechst 33342 (1:1000, Invitrogen) was added to secondary antibody staining. Sections were mounted in anti-fade mounting medium (ProLong Diamond Antifade Mountant, Invitrogen).

Bright field images were acquired on a Zeiss Axio Scan.Z1 slide scanner using a 20×/0.8 Plan-Apochromat objective lens (Zeiss). Images were cropped to individual arterial cross sections and analyzed, blinded, in ZEN (v3.2, blue edition) software. A profile ruler tool was applied to measure the media and lumen diameters (minimum diameter) of each cross section, and the ratio was calculated. Fluorescent images were acquired on an upright laser scanning confocal microscope using a 63×/1.4 Oil Plan-Apochromat objective lens (Zeiss). A tile scan (2 × 2 tiles) was used to ensure imaging of the entire arterial cross section. Mean intensity measurements were acquired in ImageJ (Fiji) (v2.1.0/1.53f/Java 1.8.0_172) by measuring the entire cross section and a region of interest (ROI; 20 × 20 μm). Measurements were converted to percentage relative to WKY control. Statistical analysis was performed in GraphPad Prism (v9) using unpaired Student t test.

Western blot analysis

Tissue extracts were dissolved in SDS-sample buffer (NuPAGE LDS Sample Buffer (4x), Thermo Scientific) containing 0.1 M DTT (NuPAGE Sample Reducing Agent (10×), Thermo Scientific) and heat-treated for 10 min at 70 °C. Proteins were separated by gel-electrophoresis on 4 to 12% Bis-Tris SDS-PAGE gels (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) nitrocellulose membranes (Immobilon-FL, Millipore). The membranes were blocked in Odyssey Blocking buffer (Li-Cor Biosciences) and incubated with primary antibodies anti-Flot1 (HPA001393, 1:1000, Sigma), anti-Fmo2 (HPA028261, 1:1000, Sigma) or anti-alpha smooth muscle cell actin (ab32575, 1:2500, Abcam) O/N at 4 °C. The membranes were washed (PBS-Tween 0.1%) and incubated with conjugated secondary antibodies (α-Rabbit 680 or 800, Li-Cor Biosciences; 1:10,000, respectively) for 1 h at RT. Proteins were visualized using an Odyssey Infrared Imaging System (Li-Cor Biosciences) and analyzed with supplier’s software (Image Studio Lite, v5.2.5). Protein bands corresponding to Flot1 and Fmo2 were normalized to their corresponding α-actin band, and intensities from SHR and WKY controls were compared. Statistical analysis was performed in GraphPad Prism (v9) using unpaired Student t test.

Sample preparation for proteomic analysis

Tissue extracts (100 μg) were diluted in digestion buffer (0.5% SDC in 50 mM TEAB), heat-treated for 5 min at 95 °C, and prepared by a modified filter-aided sample preparation (FASP) protocol (65). In brief, tissue extracts were transferred to 0.5 ml (tilted) spin filters (Amicon), centrifuged at 14,000g for 15 min, and reduced and alkylated by addition of digestion buffer containing 1:50 (v:v) tris(2-carboxyethyl)phosphine (0.5 M, Sigma) and 1:10 (v:v) 2-chloroacetamide (Sigma) for 30 min at 37 °C. Samples were digested in fresh digestion
buffer containing 1 µg Trypsin/LysC mix (Promega) and 0.01% ProteaseMAX (Promega) O/N at 37 °C. Digested samples were desalted using stage-tips containing styrene divinylbenzene reversed-phase sulfonate material (SDB-RPS; 3 M).

The mesenteric artery-based library was generated using a pooled digested and stage-tipped sample from the 12-week-old SHR and WKYs that was fractionated. A high-pH reverse-phase peptide (HpH) fractionation kit (Pierce, Thermo Scientific) was used to create the 15 fractionations.

Data acquisition by liquid chromatography–mass spectrometry (LC-MS)

Peptides were separated on 50 cm columns packed with ReproSil-Pur C18-AQ 1.9 µm resin (Dr Maisch GmbH). Liquid chromatography was performed on an EASY-nLC 1200 ultra-high-pressure system coupled through a nanoelectrospray source to an Exploris 480 mass spectrometer (Thermo Fisher Scientific). Peptides were loaded in buffer A (0.1% formic acid) and separated applying a nonlinear gradient of 5 to 65% buffer B (0.1% formic acid, 80% acetonitrile) at a flow rate of 300 nl/min over 100 min. Spray voltage was set to 2400 V. Data acquisition switched between a full scan (120,000 resolution, 45 ms max. injection time, AGC target 300%) and 49 DIA scans with isolation width of 13.7 m/z and windows overlap of 1 m/z spanning a precursor mass range of 361 to 1033 m/z (15,000 resolution, 22 ms max. injection time, AGC target 100%). Normalized collision energy was set to 27.

Protein identification by computational data analysis

Raw DDA and DIA files were first searched in Spectronaut (14.6.201001.47784) using the Pulsar search engine to generate the hybrid library. Identification settings: Digest type = specific, missed cleavage = 2, min peptide length = 7, max peptide length = 52, digestion rule = Trypsin/P; Identification settings: Peptide, protein and PSM FDR = 0.01; Spectral library filters = m/z 1800 to 300, Precursor min and max = 6 and 3, Best N fragments per peptide = True, missed cleavage = false, modification = none. Tolerance settings: Searches were set to Dynamic and Factor was set to 1. The generated hybrid library was used for library-based DIA analysis using default settings. Data were searched against UniProt FASTA database (UP000002494_10116.fasta (21,587 entries) and UP000002494_10116_additional.fasta (9981 entries), August 2020). Label-free quantification was performed in Spectronaut using default manufacturer settings.

Bioinformatic analysis of MS data

All downstream data analysis was performed in Perseus (v1.6.14.0) (66) and R (v4.0.3). Protein groups from the datasets were filtered by ≥2 unique peptides (precursors identified in Spectronaut) and minimum 75% valid values in each group. Data were log2 transformed and missing values were imputed (width = 0.2, down shift = 1.8). Volcano plots and two-sided Student t test were generated using 250 randomizations, permuted FDR <0.05, and p < 0.05. ECM enrichment was achieved by comparing with a curated matrisome gene list (18, 19) and selecting overlapping proteins for further analysis. Hierarchical clustering was based on z-scored LFQ values and generated by average linkage, preprocessing with k-means, and Euclidean distance. The z-score normalization was calculated by subtracting mean intensity from each protein value across all samples followed by division by the standard deviation. ClueGO network analysis was performed in Cytoscape (67) (v3.8.1) using the ClueGo app (17) (v2.5.7). In brief, Rattus norvegicus was selected as organism, significantly regulated proteins were added, and the Gene Ontology (GO) biological processes (GO-BiologicalProcess, CellularComponent, ImmuneSystemProcess, MolecularFunction-EBI-UniProt-GOA-ACAP-ARAP, downloaded 15.01.2021) and Kyoto Encyclopedia of Genes and Genomes (KEGG, downloaded 15.01.2021) with the kappa-score = 0.4 were used. Two-sided hypergeometric test was used with false-discovery rate (FDR) corrected for multiple testing (Bonferroni step down, p ≤ 0.05) and GO term fusion was enabled. A minimum of three genes and 4% genes per term were applied.

Data availability

MS raw files and hybrid libraries have been deposited to the ProteomeXchange Consortium via PRIDE (68) with the identifier PXD026051.

Supporting information—This article contains supporting information.

Acknowledgments—Mass spectrometry analyses were performed by the Proteomics Research Infrastructure (PRI) at the University of Copenhagen (UCPH), supported by the Novo Nordisk Foundation (NNF) (grant agreement number NNF19SA0059305).

Author contributions—J. A. B., C. A., and T. A. J. conceptualization; J. A. B. data curation; J. A. B. formal analysis; T. A. J. funding acquisition; J. A. B., C. A., and T. A. J. investigation; J. A. B., C. A., and T. A. J. methodology; T. A. J. project administration; T. A. J. resources; J. A. B. software; T. A. J. supervision; J. A. B., C. A., and T. A. J. writing—original draft; J. A. B., C. A., and T. A. J. writing—reviewing and editing.

Funding and additional information—J. A. B. was funded by a Lundbeck Foundation grant awarded to T. A. J. (grant number R323-2018-3674).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BP, blood pressure; DIA-MS, data-independent acquisition–mass spectrometry; ECM, extracellular matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MS, mass spectrometry; PCA, principal component analysis; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; VSMC, vascular smooth muscle cell; WKY, Wistar Kyoto.

References

1. NCD Risk Factor Collaboration (NCD-RisC) (2017) Worldwide trends in blood pressure from 1975 to 2015: A pooled analysis of 1479 population-based measurement studies with 19·1 million participants. Lancet 389, 37–55
Proteome of arteries in hypertension

2. Dzau, V. J., and Balbattat, C. A. (2019) Future of hypertension. Hypertension 74, 450–457
3. Heagerty, A. M., Aalkejaer, C., Bund, S. I., Korsgaard, N., and Mulvany, M. J. (1993) Small artery structure in hypertension. Dual processes of remodeling and growth. Hypertension 21, 391–397
4. Intengan, H. D., and Schiffer, E. L. (2001) Vascular remodeling in hypertension. Hypertension 38, 581–587
5. González, A., López, B., Ravassa, S., Querejeta, R., Larman, M., Diez, J., and Fortuno, M. A. (2002) Stimulation of cardiac apotopsis in essential hypertension. Hypertension 39, 75–80
6. Cai, Z., Gong, Z., Li, Z., Li, L., and Kong, W. (2021) Vascular extracellular matrix remodeling and hypertension. Antioxid. Redox Signal. 34, 765–783
7. Virdis, A., and Schiffer, E. L. (2003) Vascular inflammation: A role in vascular disease in hypertension? Curr. Opin. Nephrol. Hypertens. 12, 181–187
8. Dharmashankar, K., and Widlansky, M. E. (2010) Vascular endothelial function and hypertension: Insights and directions. Curr. Hypertens. Rep. 12, 448–455
9. Aebersold, R., and Mann, M. (2016) Mass-spectrometric exploration of proteome structure and function. Nature 537, 347–355
10. Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selesev, N., Reiter, L., Bonner, R., and Aebersold, R. (2012) Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: A new concept for consistent and accurate proteome analysis. Mol. Cell. Proteomics 11, O111.016717
11. Hatzinikos, D., Barkas, G., Critselis, E., Zoidakis, J., Gakiopoulou, H., Hatziioanou, D., Barkas, G., Critselis, E., Zoidakis, J., Gakiopoulou, H., Klyueva, N. Z. (2013) The changes in metabolism of the regulatory brain dentates: Genetic and environmental factors. Citterio, L., Simonini, M., Brioni, E., Magnaghi, C., Colombo, G. I., Santini, G., Nistri, F., Cellai, F., Lenti, S., et al. (2020) Hypertension in high school students: Genetic and environmental factors. Hypertension 75, 71–78
21. Cheng, Y., Sun, D., Zhu, B., Zhou, W., Lv, C., Kou, F., and Wei, H. (2020) Integrative metabolic and proteomic profiling of the brainstem in spontaneously hypertensive rats. J. Proteome Res. 19, 4114–4124
22. Decharatchakul, N., Settasatian, C., Settasatian, N., Komansan, N., Kukongviriyapan, U., Intharapetch, P., Senthong, V., and Sawanyawisuth, K. (2020) Association of combined genetic variations in SOD3, GPX3, PON1, and GSTT1 with hypertension and severity of coronary artery disease. Heart Vessels 35, 918–929
23. Rapp, J. P. (1987) Use and misuse of control strains for genetically hypertensive rats. Hypertension 10, 7–10
24. Oram, J. F., and Yokoyama, S. (1996) Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. J. Lipid Res. 37, 2473–2491
25. Larsson, M., Vorsjö, E., Talmud, P., Lookene, A., and Olvecreona, G. (2013) Apolipoproteins C-I and C-III inhibit lipoprotein lipase activity by displacement of the enzyme from lipid droplets. J. Biol. Chem. 288, 33997–34008
26. Zicha, J., Pechánová, O., Caçaynová, S., Cebová, M., Krítek, F., Tóroik, J., Simko, F., Dohosová, Z., and Kunes, J. (2006) Hereditary hypertriglyceridemic rat: A suitable model of cardiovascular disease and metabolic syndrome? Physiol. Res. 55, 549–563
27. Lin, Y.-P., Hsu, M.-E., Chiou, Y.-Y., Hsu, H.-Y., Tsai, H.-C., Peng, Y.-J., Lu, C.-Y., Pan, C.-Y., Yu, W.-C., Chen, C.-H., Chi, C.-W., and Lin, C.-H. (2010) Comparative proteomic analysis of rat aorta in a subnational nethyroid proteome model. Proteomics 10, 2429–2443
28. Chen, Y.-Q., Zhou, H.-M., Chen, F.-F., Liu, Y.-P., Han, L., Song, M., Wang, Z.-H., Zhang, W., Shang, Y.-Y., and Zhong, M. (2020) Testosterone ameliorates vascular aging via the Gas6/Axl signaling pathway. Aging (Albany N. Y.) 12, 16111–16125
29. Keranov, S., Dörr, O., Jafari, L., Liebetrau, C., Keller, T., Troidl, C., Krücbach, S., Voss, S., Richter, M., Tello, K., Gall, H., Ghofrani, H. A., Mayer, E., Seeger, W., Hamm, C. W., et al. (2020) SPARCL1 as a biomarker of maladaptive right ventricular remodelling in pulmonary hypertension. Biomarkers 25, 290–296
30. Lord, M. S., Melrose, J., Day, A. J., and Whitlock, J. M. (2020) The intera- trypsin inhibitor family: Versatile molecules in biology and pathology. J. Histochem. Cytochem. 68, 907–927
31. Robaczewska, J., Kedziora-Kornatowska, K., Kozakiewicz, M., Zary-Sikorska, E., Pawluk, H., Pawlizak, W., and Kedziora, J. (2016) Role of glutathione metabolism and glutathione-related antioxidant defense systems in hypertension. J. Physiol. Pharmacol. 67, 331–337
32. Muda, P., Kampus, P., Zilmer, M., Zilmer, K., Kairane, C., Ristimäe, T., Fischer, K., and Teesalu, R. (2003) Homocysteine and red blood cell glutathione as indices for middle-aged untreated essential hypertension patients. J. Hypertens. 21, 2329–2333
33. Kerr, S., Brosnan, M. J., McIntyre, M., Reid, J. L., Dominiczak, A. F., and Hamilton, C. A. (1999) Superoxide anion production is increased in a model of genetic hypertension. Hypertension 33, 1353–1358
34. He, N. G., Awasthi, S., Singhal, S. S., Trent, M. B., and Boor, P. J. (1998) The role of glutathione S-transferases as a defense against reactive electrophiles in the blood vessel wall. Toxicol. Appl. Pharmacol. 152, 83–89
35. Delles, C., Padmanabhan, S., Lee, W. K., Miller, W. H., McBride, M. W., McClure, J. D., Brain, N. J., Wallace, C., Marzano, A. C. B., Schmieder, R. E., Brown, M. J., Caulfield, M. J., Munroe, P. B., Farrall, M., Webster, J., et al. (2008) Glutathione S-transferase variants and hypertension. J. Hypertens. 26, 1343–1352
36. Negri, C., Fiorillo, C., Marchetti, E., Pacini, A., Liguri, G., and Nassi, P. (2006) Glutathione S-transferase variants and hypertension. J. Hypertens. 26, 1343–1352
37. Negri, C., Cattini, C., Alvis-Lopes, R., Rios, F. J., Camargo, L. L., Naganostopoulos, A., Arner, A., and Montezano, A. C. (2018) Vascular...
smooth muscle contraction in hypertension. *Cardiovasc. Res.* **114**, 529–539

42. Zhu, Y., Qu, J., He, L., Zhang, F., Zhou, Z., Yang, S., and Zhou, Y. (2019) Calcium in vascular smooth muscle cell elasticity and adhesion: Novel insights into the mechanism of action. *Front. Physiol.* **10**, 852

43. Kettenhofen, R., Meyer zu Brickwedde, M.-K., Hans Vetter, Y. K., and Zacharius, T. (2012) Abnormalities of membrane function and lipid metabolism in hypertension: A review. *Annu. J. Hypertens.* **15**, 315–331

44. Cha, J. H., Lee, H. R., Kim, K. C., Cho, M.-S., and Hong, Y. M. (2012) Calcium in vascular oxidative stress and hypertension. *Cardiovasc. Res.* **95**, 928–934

45. Otto, G. P., and Nichols, B. J. (2011) The roles of flotillin microdomains - endocytosis and beyond. *J. Cell Sci.* **124**, 3933–3940

46. Zicha, J., Kunes, J., and Devynck, M. (1999) Abnormalities of membrane function and lipid metabolism in hypertension: A review. *Ann. J. Hypertens.* **12**, 315–331

47. Otto, G. P., and Nichols, B. J. (2011) The roles of flotillin microdomains - endocytosis and beyond. *J. Cell Sci.* **124**, 3933–3940

48. Banning, A., Babuke, T., Kurre, N., Meister, M., Ruonala, M., and Tikkanen, R. (2018) Flotillins regulate focal adhesions by interacting with α-actinin and by influencing the activation of focal adhesion kinase. *Cells* **7**, 28

49. Soni, S., Raaijmakers, A. J. A., Raaijmakers, L. M., Damen, J. M. A., van Stuijvenberg, L., van, M. A., Heck, A. J. R., van Veen, T. A. B., and Scholten, A. (2016) A proteomics approach to identify new putative cardiac intercalated disc proteins. *PLoS One* **11**, e0152231

50. Kessler, E. L., van Stuijvenberg, L., van Bavel, J. J. A., van Bennekom, J., Zwartsen, A., Rivaud, M. R., Vink, A., Ephrin, I. R., Postma, A. V., van Tintelen, J. P., Remme, C. A., Vos, M. A., Banning, A., de Boer, T. P., Tikkanen, R., et al. (2019) Flotillins in the intercalated disc are potential modulators of cardiac excitability. *J. Mol. Cell. Cardiol.* **126**, 86–95

51. Liu, X., Du, H., Chen, D., Yuan, H., Chen, W., Jia, W., Wang, X., Li, X., and Gao, L. (2019) Cyclophilin D deficiency protects against the development of mitochondrial ROS and cellular inflammation in aorta. *Biochem. Biophys. Res. Commun.* **508**, 1202–1208

52. Itani, H. A., Dikalova, A. E., McMaster, W. G., Nazarewicz, R. R., Bikiyevyata, A. T., Harrison, D. G., and Dikalov, S. I. (2016) Mitochondrial Cyclophilin D in vascular oxidative stress and hypertension. *Hypertension* **67**, 1218–1227

53. Crevpe, C., Malinouskaya, L., Volvert, M.-L., Gillard, M., Close, P., Malaise, O., Laguette, S., Cornez, I., Rahmouni, S., Ormenese, S., Belachew, S., Malgrange, B., Chapelle, J.-P., Siebenlist, U., Moonen, G., et al. (2009) Elongator controls the migration and differentiation of cortical neurons through acetylation of α-tubulin. *Cell* **136**, 551–564

54. Cheishvili, D., Maayan, C., Cohen-Kupiec, R., Leifer, S., Weil, M., Ast, G., and Razin, A. (2011) IKAP/Elp1 involvement in cytoskeleton regulation and implication for familial dystonia. *Hum. Mol. Genet.* **20**, 1585–1594

55. Lindman, J., Khammy, M. M., Lundegaard, P. R., Aalkjaer, C., and Jepps, T. A. (2018) Microtubule regulation of Kv7 channels orchestrates cAMP-mediated vasorelaxations in rat arterial smooth muscle. *Hypertension* **71**, 336–345

56. van der Horst, J., Salmoné, R., Abbott, G. W., Ozhathil, L. C., Hagglund, P., Barrese, V., Chuang, C. Y., Jespersen, T., Davies, M. I., Greenwood, I. A., Gourdon, P., Aalkjaer, C., and Jepps, T. A. (2021) Dynemin regulates Kv7.4 channel trafficking from the cell membrane. *J. Gen. Physiol.* **153**, e202012760

57. Hashimoto-Komatsu, A., Hirase, T., Asaka, M., and Node, K. (2011) Angiotensin II induces microtubule reorganization mediated by a deacetylase SIRT2 in endothelial cells. *Hypertens. Res.* **34**, 949–956

58. Mehboob, M. Z., and Lang, M. (2021) Structure, function, and pathology of protein O-glucosyltransferases. *Cell Death Dis.* **12**, 71

59. Morris, H. E., Neves, K. B., Montezano, A. C., MacLean, M. R., and Tous, R. M. (2019) Notch3 signalling and vascular remodelling in pulmonary arterial hypertension. *Clin. Sci.* **133**, 2481–2498

60. Myllyharju, J. (2003) Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol.* **22**, 15–24

61. Myllyharju, J., and Schipani, E. (2010) Extracellular matrix genes as hypoxia-inducible targets. *Cell Tissue Res.* **339**, 19–29

62. Galant, C., Marchandise, J., Stoenoiu, M. S., Ducieux, J., De Groof, A., Pirene, S., Van den Eynde, B., Houssiau, F. A., and Lauwers, B. R. (2019) Overexpression of ubiquitin-specific peptidase 15 in systemic sclerosis fibroblasts increases response to transforming growth factor β. *Rheumatology* **58**, 708–718

63. Schiffrin, E. L. (2012) Vascular remodeling in hypertension. *Hypertension* **59**, 367–374

64. van der Horst, J., Manville, R. W., Hayes, K., Thomsen, M. B., Abbott, G. W., and Jepps, T. A. (2020) Acetaminophen (paracetamol) metabolites induce vasodilation and hypotension by activating Kv7 potassium channels directly and indirectly. *Arterioscler. Thromb. Vasc. Biol.* **40**, 1207–1219

65. Wiśniewski, J. R., Zougman, A., Nagaraj, N., and Mann, M. (2009) Universal sample preparation method for proteome analysis. *Nat. Methods* **6**, 359–362

66. Tyanova, S., Temu, T., Sinticryn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, I. (2016) The Perseus computational platform for comprehensive analysis of (pro)teomics data. *Nat. Methods* **13**, 731–740

67. Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., and Trey, I. (2003) Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498–2504

68. Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundra, D. I., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., Pérez, E., Uszkoreit, J., Pfeuffer, J., Sachsenberg, T., Yilmaz, Ş., et al. (2019) The PRIDE database and related tools and resources in 2019: Improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450