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# Altered Liver Gene Expression Due to Hypertension and Age in Rats

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## Abstract

Hypertension and metabolic syndrome, both of which increase with age, are multifactorial disorders. Their etiology is complex, making it challenging to isolate involved genes. This study aimed to characterize the hepatic gene expression in spontaneously hypertensive rats (SHR) at different ages. Blood pressure in SHR was determined by tail-cuff method at one and three months of age. Hepatic RNA was isolated and gene expression was compared using microarrays. Comparison between SHR and normotensive rats revealed significant variation in gene expression: 98 genes were upregulated and 122 were downregulated in SHR; while 88 genes were upregulated and 139 genes were downregulated in age-matched normotensive rats. Furthermore, within the SHR group, 110 genes were found to be upregulated and 168 genes downregulated across different ages. Analyses via the Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes pathways revealed that several genes are potentially implicated in both, hypertension and metabolic syndrome. The results suggest that SHR display variations in gene expression due to aging, and when compared to normotensive rats. These variations could contribute to the development of hypertension and metabolic syndrome. Microarray studies involving older rats are necessary to further validate these findings.

## Keywords

Hypertension, Metabolic Syndrome, Gene Expression, SHR, Microarray

## 1. Introduction

Hypertension and metabolic syndrome (MetS) are diseases of multifactorial origin, that significantly contribute to high morbidity and mortality around the world [1] [2]. MetS is characterized by central obesity, impairment of lipid metabolism, hyperglycemia/insulin resistance, and hypertension [3]. Primary hypertension, a component of MetS, is prevalent globally and is a major factor risk for cardiovascular diseases (CVDs) [4]. Hypertension involves abnormal responses in the central nervous, cardiovascular, and renal systems. In contrast, MetS is a cluster of metabolic and cardiovascular symptoms, including hyperglycemia, abdominal obesity, dyslipidemia, and dysfunctions of the liver, pancreas, and adipose tissue [5]. Both MetS and hypertension are known to increase with age, leading to the common perception of these conditions as age-related disorders [2] [6] [7].

In recent years there have been significant advances in genetic mapping of both hypertension and MetS [1] [8] [9] [10] [11]. However, the multifactorial nature of these conditions complicates to isolate genes involved in their etiology. To address this, two approaches have proven valuable in the search for underlying diseases factors: 1) due to their similar pathophysiology, spontaneously hypertensive rats (SHR) serve as a widely used animal model for human primary hypertension and metabolic syndrome [12] [13] [14] [15] [16]; and 2) microarrays, a robust methodology for study expression of thousands of genes in a single experiment [8] [16] [17], allow for the comprehensive analysis of gene expression in these models of hypertension/MetS. This understanding of the expression in SHRs can provide useful information about genes and pathways related to pathophysiological traits in humans. Prompted by these insights, we aimed to characterize the gene expression profiles of SHR liver at different stages of hypertension.

## 2. Materials and Methods

### 2.1. Animals and Ethical Statement

Male spontaneously hypertensive rats (SHR, aged 1 month as prehypertensive stage, and 3 months as hypertensive stage, $n = 2$), and Wistar Kyoto rats (WKY, aged 1 month, and 3 months, both ages as normotensive stage, $n = 2$), used as a control group, were obtained from the animal facility of the Institute of Cell Physiology at the National Autonomous University of Mexico. All animal housing, care, and procedures were conducted in accordance with Mexican Regulations for Animal Care and Use (NOM-062-ZOO-1999, SAGARPA, Mexico), and the Guide for the Care and Use of Laboratory Animals, as promulgated by the U.S. National Institutes of Health (8$^{th}$ edition, 2011). Animals were maintained in a pathogen-free environment under controlled conditions (22˚C ± 2˚C, 40% - 60% humidity, 12 hours light/12 hours dark cycle), with free access to tap water, and fed a standard rat chow *ad libitum* throughout the experimental periods, except during the overnight (12 - 16 h) fasting period before being euthanized.

All experimental protocols were approved by the Ethics Committee of the Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (Protocol number 1368).

## 2.2. Procedures

### 2.2.1. Measurement of Blood Pressure in SHR and WKY Rats

The indirect measurement of blood pressure was carried out using a tail-cuff device (Automatic Blood Pressure Computer, Model LE 5007; Letica, Panlab, Spain), as described previously [18]. Briefly, rats were gently restrained in a size-appropriate plastic container. A blood pressure transducer and a ring containing inflatable latex were placed on the tail, while the rat was kept warm within the device (35˚C - 37˚C). The rats were trained to remain inside the container with the cuff on the tail, and to tolerate inflation and deflation of the latex ring (this process was repeated several times beforehand). Each rat then underwent a minimum of three blood pressure measurements. This procedure was conducted between 8 and 10 AM.

### 2.2.2. Tissue Collection

Rats were sacrificed by cervical dislocation, after which their livers were rapidly removed, rinsed in phosphate buffer solution at 4˚C, and frozen in cryogenic vials at −80˚C until RNA extraction.

### 2.2.3. RNA Isolation

Total RNA was extracted from 50 mg of the frozen livers' samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), as per the manufacturer's protocol. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm using an Agilent™ Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), ensuring all ratios exceeded 1.8. The integrity of the RNA was confirmed through electrophoresis on a 1.5% denaturing agarose gel.

### 2.2.4. Gene Expression Profiling in SHR and WKY Rats

Gene expression in liver samples from SHR and WKY rats was analyzed using rat microarrays. Three independent experiments were conducted: the first microarray compared the gene expression profile of 1-month-old SHR with age-matched WKY; the second microarray compared the gene expression profile of 3-month-old SHR with age-matched WKY; and the third microarray compared the gene expression profile of 1-month-old with 3-month-old SHR.

### 2.2.5. Arrays Printing

The *Rattus norvegicus* 70-mer oligo library from OPERON Oligo Sets (http://omad.operon.com/), containing 5000 gene-specific oligonucleotide probes representing the better-known genes, were suspended in 40 μM of Micro Spotting solution (TeleChem International Inc., Portland, OR, USA). SuperAmine-coated slides (25 × 75 mm, TeleChem) were printed in duplicate and fixed

at 80˚C for 4 hours. For pre-hybridization, the slides were re-hydrated with water vapor at 60˚C, then fixed with two cycles of UV light (1200J). After being boiled for 2 min at 92˚C, the slides were washed with 95% ethanol for 1 min and pre-hybridized in 5X SSC, 0.1% SDS, and 1% BSA for 1 hour at 42˚C. The slides were then washed and dried for further hybridization.

#### 2.2.6. Probe Preparation and Hybridization to Arrays

Ten µg of total RNA were used for complementary DNA (cDNA) synthesis, which incorporated dUTP-Alexa555 or dUTP-Alexa647, employing the First-Strand cDNA Labeling Kit (Invitrogen). The incorporation of a fluorophore was analyzed by measuring the absorbance at 555 nm for Alexa555, and 650 nm for Alexa647. Equal quantities of labeled cDNA were hybridized using the UniHyb hybridization solution (TeleChem). The arrays were incubated for 14 h at 42˚C, then washed three times with 1X SCC and 0.05% SDS at room temperature.

#### 2.2.7. Data Acquisition and Analysis of Array Images

The acquisition and quantification of array images were performed on a GenePix 4100A with its accompanying software, GenePix (Molecular Devices, Sunnyvale, CA, USA); all images were captured at a resolution of 10 µm. For each spot, the Alexa555 and Alexa647 density mean values, as well as the background mean values, were calculated using the ArrayPro Analyzer software (Media Cybernetics, Rockville, MD, USA).

#### 2.2.8. Functional Analysis by Gene Ontology and Gene Pathways

Microarray data analysis was carried out using the genArise software, developed in our Informatics Unit (http://www.ifc.unam.mx/genarise/). GenArise performs several operations, including background correction, lowess normalization, intensity filtering, replicates analysis, and selecting differentially expressed genes. The goal of genArise is to identify genes that show substantial evidence of differential expression. The Database for Annotation, Visualization, and Integrated Discovery (DAVID Bioinformatics database) was employed to identify functionally related genes categories. Differentially expressed genes were considered significant and selected based on a change in expression of at least 1.5-fold, and a p-value of ≤0.05. The DAVID software analyzed significant enrichment of differentially expressed genes within Gene Ontology (GO) terms, and involves assessment of advanced pathway analysis [19]. Biological pathways were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) [20]. KEGG pathway analysis (http://www.kegg.jp/kegg/docs/statistics.html) is a comprehensive pathway prediction tool that contains >200 pathways, and a collection of pathway maps representing molecular interaction and reaction networks for sequences. The microarray dataset has been deposited in the NCBI Gene Expression Omnibus (GEO) public database in compliance with MIAME (Minimum Information About a Microarray Experiment) guidelines (GEO series accession number GSE96587).

### 2.3. Statistical Analysis

Statistical analysis was performed using commercially available GraphPad Prism version 4.0 software (La Jolla, CA, USA). The data are expressed as the mean ± SEM. Statistical significance was determined using Student's *t*-test, with the significance set at $p \leq 0.05$.

## 3. Results

### 3.1. Body Weight Measurement in SHR and WKY Rats

The body weights were 83 ± 1.9 g in WKY *vs.* 64.7 ± 1.3 g in SHR at 1-month-old ($p \leq 0.05$), while 310.1 ± 3.3 g in WKY *vs.* 251.9 ± 4 g in SHR at 3-month-old ($p \leq 0.05$).

### 3.2. Systolic Blood Pressure in SHR and WKY Rats

The systolic blood pressure of SHR was similar to that of WKY rats at 1 month-old (113 ± 2 mmHg in WKY *vs.* 112 ± 3 mmHg in SHR at pre-hypertensive stage). However, it was higher at 3-month-old (112 ± 3 mmHg in WKY *vs.* 184 ± 4 mmHg in SHR at the hypertensive stage). Blood pressure increased in SHR from 1- to 3-month-old ($p \leq 0.5$), whereas it did not change with age in WKY. The study model (SHR) emulates the phenomenon observed in humans, where blood pressure increases with age.

### 3.3. Gene Expression Profiles in SHR and WKY Rats

Three independent microarray analyses were performed. The first examined gene expression in the liver of 1-month-old SHR *vs.* age-matched WKY. The second assessed the gene expression of 3-month-old SHR *vs.* age-matched WKY. The third compared the gene expression profile of 1-month-old *vs.* 3-month-old SHR. The hierarchical clusters show the differential expression of all the transcripts; a color scale is included with the heat map to visualize the consistency of the expression patterns within each group of samples (**Figure 1(a)**), as well as the difference between groups (WKY *vs.* SHR). Additionally, a Venn diagram shows the total number of genes regulated up and down in the different microarrays and the number of common genes found (**Figure 1(b)**).

### 3.4. Microarrays Gene Ontology and Gene Pathways

Data from the microarrays were analyzed using the DAVID database software to identify genes differentially expressed in SHR, compared with WKY at both ages ($p \leq 0.05$). All identified differences are shown in **Table 1** and **Table 2**.

Pathway analysis was conducted through the DAVID bioinformatics database to identify biological processes. The most enriched databases of pathways from our study were obtained through KEGG, as shown in **Table 3**.

Gene Ontology enrichment analysis is based on the knowledge of various biological elements, and KEGG annotations contain information on more than 200 pathways. The identified GO functions were predominantly associated with

adrenergic signaling, renin secretion, aldosterone synthesis and secretion, and calcium signaling in SHR rats. The KEGG pathway analysis revealed differentially expressed genes highlighted with red stars, as demonstrated in a representative selected pathway, *i.e.*, aldosterone synthesis and secretion (Figure 2).

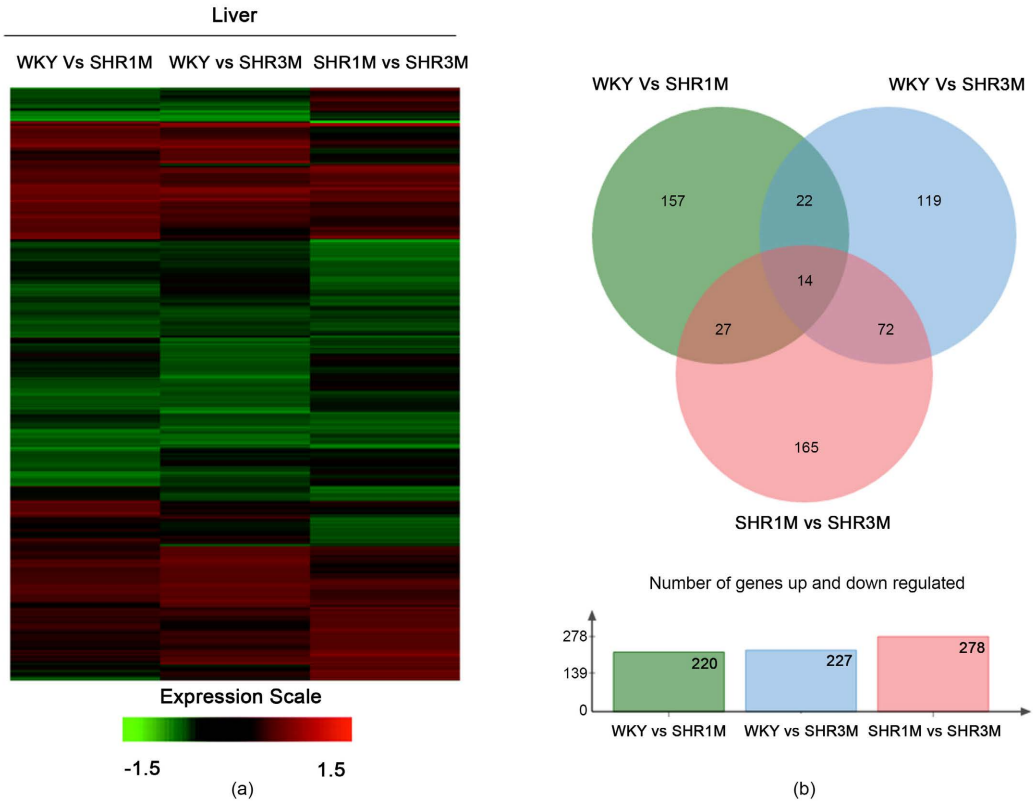


Figure 1. (a) Hierarchical cluster analysis of SHR and WKY rats' microarrays; (b) Venn diagram showing the total number of genes regulated up and down in the microarrays, and the number of genes in common.

Table 1. Differentially expressed gene transcripts in the liver of 1- and 3-month-old SHR *vs.* age-matched WKY.

| Number of genes | 1 month-old | 3 month-old | Common in both |
|---|---|---|---|
| Up-regulated | 98 | 88 | 12 |
| Down-regulated | 122 | 139 | 10 |
| Total | 220 | 227 | 22 |

$p \leq 0.05$, fold change ≤1.5 or >1.5.

Table 2. Differentially expressed gene transcripts in the liver of 1-month-old *vs.* 3-month-old SHR.

| | Number of genes | Common SHR 1-month-old *vs* WKY | Common SHR 3-month-old *vs* WKY |
|---|---|---|---|
| Up-regulated | 110 | 16 | 19 |
| Down-regulated | 168 | 7 | 34 |
| Total | 278 | 23 | 53 |

$p \leq 0.05$, fold change ≤1.5 or >1.5.

**Table 3.** List of selected biological pathways with differentially expressed genes in 1- and 3-month-old SHR *vs.* age-matched WKY.

| Description | Genes | p-value | Direction of change |
|---|---|---|---|
| Adrenergic signaling | 12 | 7.2E-5 | Up in 1-month-old SHR |
| | 13 | 2.9E-5 | Up in 3-month-old SHR |
| Calcium signaling pathway | 9 | 2.9E-5 | Up in 1-month-old SHR |
| cAMP signaling pathway | 9 | 2.7E-2 | Up in 1-month-old SHR |
| | 11 | 2.0E-3 | Up in 3-month-old SHR |
| Pancreatic secretion | 6 | 3.2E-2 | Up in 1-month-old SHR |
| | 9 | 4.6E-4 | Up in 3-month-old SHR |
| Endocrine and other factor-regulated calcium reabsorption | 4 | 5.5E-2 | Up in 1-month-old SHR |
| | 4 | 6.0E-2 | Up in 3-month-old SHR |
| | 6 | 4.5E-3 | Down in 1-month-old SHR |
| TNF*α* signaling pathway | 6 | 5.0E-2 | Up in 1-month-old SHR |
| | 6 | 5.6-2 | Up in 3-month-old SHR |
| Insulin secretion | 5 | 7.3E-2 | Up in 1-month-old SHR |
| | 7 | 5.8E-3 | Up in 3-month-old SHR |
| Renin secretion | 7 | 1.6E-3 | Up in 3-month-old SHR |
| Glucagon signaling pathway | 6 | 4.2E-2 | Up in 3-month-old SHR |
| HIF-1 signaling pathway | 6 | 4.8E-2 | Up in 3-month-old SHR |
| Aldosterone synthesis and secretion | 5 | 7.6E-2 | Up in 3-month-old SHR |
| | 7 | 1.6E-2 | Down in 1-month-old SHR |
| Drug metabolism CYP450 | 6 | 2.4E-2 | Down in 1-month-old SHR |
| PPAR | 6 | 3.3E-2 | Down in 1-month-old SHR |
| Regulation of lipolysis | 5 | 2.4E-2 | Up in 3-month-old SHR |
| | 5 | 4.8E-2 | Down in 1-month-old SHR |

## 4. Discussion

Hypertension and MetS result from the interaction of multiple genetic and environmental factors [2] [14] [16], while aging is considered an essential factor for functional changes in the cardiovascular system [7] [14]. Additionally, functional changes in this system are characterized by increased blood stiffness of blood vessels, endothelial dysfunction, and subsequent systolic hypertension. These alterations are regulated by signaling pathways activated by endogenous and exogenous factors [5] [21]. On the other hand, non-alcoholic fatty liver disease (NAFLD), representing MetS, is a common comorbidity alongside hypertension in humans. Accordingly, the liver plays a crucial role in the pathophysiology of hypertension in SHR. The enzymes glutathione S-transferase μ1, sulfite oxidase, and quinonoid-dihydropterin reductase exhibit either varying protein abundance in SHR or different post-translational modifications, suggesting deviation

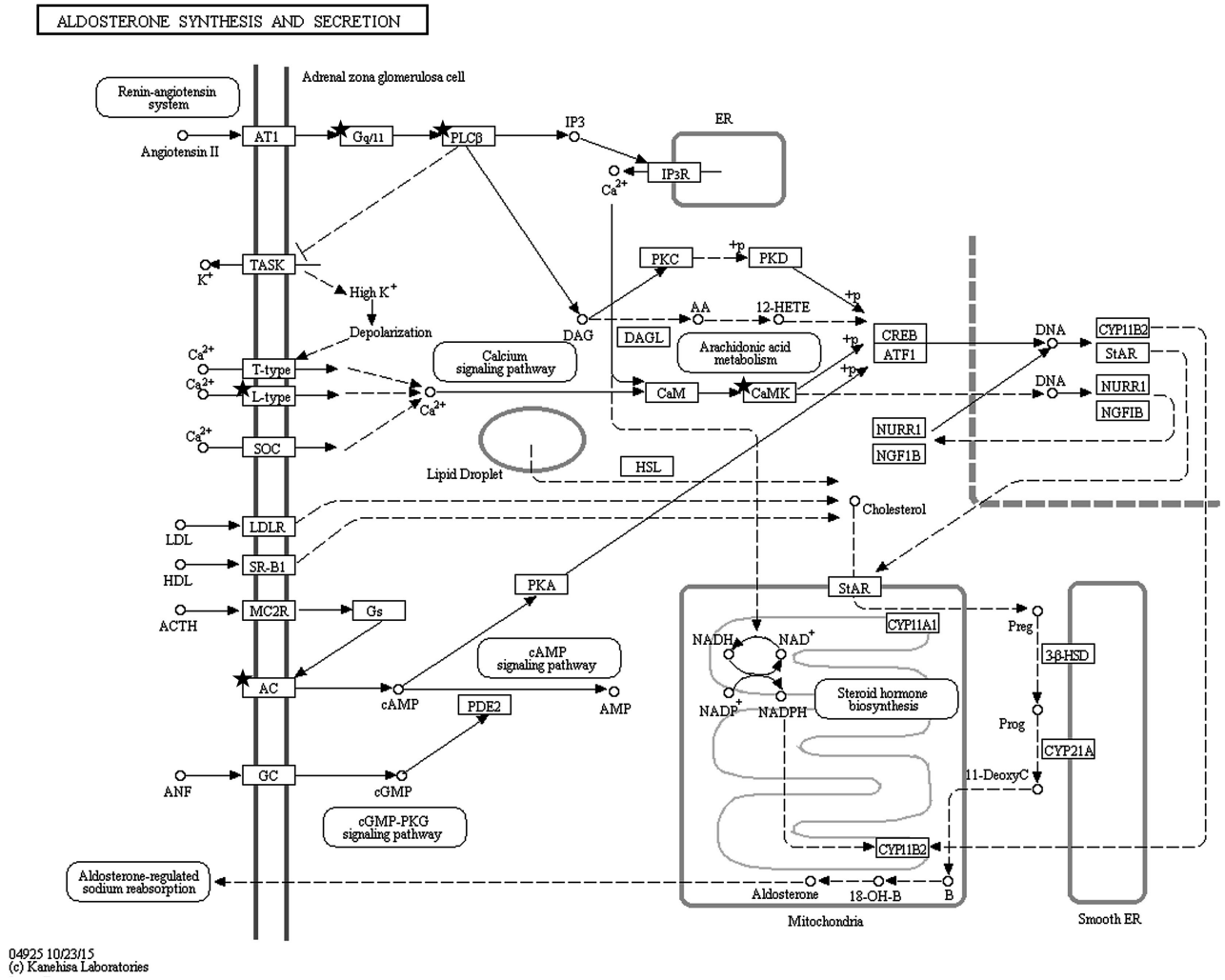


**Figure 2.** Aldosterone synthesis and secretion pathway. KEGG-pathway analysis determined by DAVID Bioinformatics resources showing differentially up-expressed genes highlighted with red stars in 3-month-old SHR *vs.* age-matched WKY.

associated with increased oxidative stress and damage in the liver of SHR compared to WKY [22]. These changes in protein expression provide evidence that hepatic physiology is altered in the SHR, and suggest novel mechanisms for the generation of reactive oxygen species, indicating that hypertension and MetS should not be treated as separate pathologies [22].

The comparison of 1- and 3-month-old SHR *vs.* age-matched WKY identified differentially expressed genes. However, it resulted in a similar number of up and down-regulated genes. Furthermore, 1-month-old *vs.* 3-month-old SHR share the expression of common genes, indicating that the global expression patterns at these two ages of hypertension are similar. Since SHR are derived from WKY, they share multiple genes. However, their phenotypes are different since SHR develops hypertension at early age and keeps this trait throughout their lives. Along with aging, SHR also shows insulin resistance/hyperinsulinemia, hypertriglyceridemia and hypercholesterolemia, conditions not observed in WKY rats (https://www.criver.com/files/pdfs/rms/rm_rm_d_animal_models_of_disease.aspx).

Pathway analysis identified the biological processes associated with differential expression in SHR rats at both ages. These biological processes included adrenergic signaling, calcium signaling, cAMP signaling, pancreatic secretion, endocrine and other factor-regulated calcium reabsorption, insulin secretion, insulin signaling, TNF-$\alpha$ signaling, renin secretion and aldosterone synthesis and secretion. A notable finding was that the network analysis revealed common genes in different biological pathways, one of these is Gq. Hormones that act through GPCRs coupled to Gq protein, such as angiotensin II (Ang II), the primary effector molecules of the renin-angiotensin system (RAS), induce cardiovascular hypertrophy through the AT1 receptor [18] [23].

The classical RAS includes renin, an enzyme catalyzing the conversion of angiotensinogen to angiotensin (Ang) I, followed by angiotensin-converting enzyme (ACE) cleavage of Ang I to II, and activation of AT1 receptors responsible for all RAS biological actions [18] [24]. The RAS was classically described as a circulatory hormonal system; however, this perception changed with the discovery of the local RAS. The RAS is relevant in the cellular domain, where individual cells have a complete system termed the intracellular RAS. From cells to tissues to the entire organism, RAS exhibits continuity while maintaining independent control at different levels. The intracellular RAS is a relatively new concept within the RAS. The local RAS is essential for regulating tissue/organ functions with clinical implications via autocrine, paracrine, or intracrine actions [25]. The local hepatic RAS is not well-defined, though studies about RAS involvement in hepatic diseases suggest a role for this system in the liver [26]. Furthermore, Ang-(1-7) has been shown to decrease liver gluconeogenesis, and the Mas receptor is a critical component of the insulin receptor signaling pathway [27].

In addition to its functions in the synthesis of the hypertensive-associated components, the hepatic RAS contributes to the pathophysiology of liver diseases, including enhanced fibrosis, sinusoidal capillarity, and increased hepatic vascular resistance, eventually resulting in portal hypertension [26] [27]. Up-regulation of RAS components, including angiotensinogen, renin, ACE, Ang II and AT1 receptor, has been reported in experimental and clinical liver injury studies, pointing out a role for this system in hepatic fibrosis and cirrhosis [28] [29].

One of the most enriched pathways was the aldosterone synthesis and secretion pathway, in which Ang II, $K^+$, and adrenocorticotropin (ACTH) are the central extracellular stimuli regulating aldosterone secretion [30]. Insufficient aldosterone secretion can lead to hypotension and circulatory shock; conversely, excessive aldosterone or high sodium can cause hypertension and exacerbate the effects of high blood pressure on multiple organs [24]; it is known that less than 15 ng/dl is considered a normal concentration in healthy individuals [31].

The effects of Ang II in the liver extended beyond its vascular bed vasoconstriction. In addition to activating AT1 receptors, Ang II acts as a pro-inflammatory

and pro-fibrotic mediator; while Ang-(1-7) appears to exert opposite effects in liver tissue, comparable to heart and kidney effects [29]. Furthermore, many genes of adrenergic signaling were up-regulated, including the $\beta$2-adrenergic receptor ($\beta$2-AR), which could be associated with their increase during aging [32]. Moreover, endogenous adrenergic catecholamines (adrenaline and noradrenaline) are critical regulators of cardiovascular functions, through the adrenergic receptor family, which belongs to GPCRs. Our results can be explained as $\beta$2-AR is expressed in the liver; hence, increased hepatic $\beta$-ARs signaling may augment fat accumulation and gluconeogenesis in the liver and its dysfunction, which might contribute to insulin resistance-related metabolic disorders and increase blood triglycerides. Previous studies have noted an association between NAFLD and age in humans and rodents, but the mechanisms leading to age-associated hepatic fat accumulation remain unknown [33] [34]. These data are consistent with the changes we observed in several genes related to the insulin secretion pathway. It is known that $\beta$-AR-mediated insulin resistance in the liver, increases in fasting hepatic glucose output, and glycemia could contribute to glucose dysregulation and diabetes developed during aging [34].

Interestingly, we found novel genes that might be related to hypertension: cytochrome P450 (CYP: CYP2C38, CYP2C39, CYP4A10, CYP11A, CYP19, and CYP21) were down-regulated. The cytochromes P450 are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids [35]. Functional and biochemical data have suggested a role for the cytochrome P450 arachidonate monooxygenases in the pathophysiology of hypertension [35] [36]. Previous studies have revealed that arachidonic acid is metabolized by the CYP enzymes of the 4A and 4F families to 20-hydroxyeicosatetraenoic acid (20-HETE), and by CYP2C and CYP2J pathways to epoxyeicosatrienoic acids (EETs), and these compounds influence both renal tubular transport and renal and peripheral vascular tone [31]. The renal formation of 20-HETE and EETs is altered in the various models of hypertension.

These findings should be used to improve the clinical diagnosis of hypertension and could be a tool to propose new therapeutic strategies for the treatment of hypertension. Our study also found differential expression of genes involved in calcium signaling, cAMP signaling, pancreatic secretion, insulin signaling and secretion, and renin secretion.

## 5. Conclusion

SHR shows significant differences in the expression of specific genes due to aging, involving complex biological networks that possibly contributed to hypertension and MetS. Future prospective studies should consider a microarray approach at older ages.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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