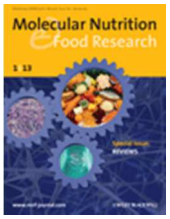

Hydroxytyrosol and its complex forms (secoiridoids) modulate aorta and heart proteome in healthy rats: potential cardio-protective effects

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Complete List of Authors:	Catalán, Úrsula; Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Sant Llorenç, 21, 43201, Reus, Spain Rubió, Laura; Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Sant Llorenç, 21, 43201, Reus, Spain López de las Hazas, Maria-Carmen; Food Technology Department, Universitat de Lleida-AGROTECNIO Center, Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain Herrero, Pol; Centre for Omic Sciences, Universitat Rovira i Virgili (COS-URV), Reus, Spain Nadal, Pedro; Centre for Omic Sciences, Universitat Rovira i Virgili (COS-URV), Reus, Spain Canela, Núria; Centre for Omic Sciences, Universitat Rovira i Virgili (COS-URV), Reus, Spain Pedret, Anna; Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Sant Llorenç, 21, 43201, Reus, Spain Motilva, Maria-José; Food Technology Department, Universitat de Lleida-AGROTECNIO Center, Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain

	SOLA, Rosa; Unitat de Recerca en Lípids i Arteriosclerosis (CIBERDEM), Hospital Universitari St. Joan de Reus, IISPV, Universitat Rovira i Virgili,
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Hydroxytyrosol and its complex forms (secoiridoids) modulate aorta and heart proteome in healthy rats: potential cardio-protective effects

Úrsula Catalán[1*], Laura Rubió [1, 2*], Maria-Carmen López de las Hazas[2], Pol Herrero[3], Pedro Nadal[3], Núria Canela[3], Anna Pedret[1], Maria-José Motilva[2†] and Rosa Solà[1†]

[1] Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Sant Llorenç, 21, 43201, Reus, Spain

[2] Food Technology Department, Universitat de Lleida-AGROTECNIO Center, Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain

[3] Centre for Omic Sciences, Universitat Rovira i Virgili (COS-URV), Reus, Spain

*These authors contributed equally to this work.

†Corresponding authors:

Rosa Solà, MD, PhD

Unitat de Recerca de Lípids i Arteriosclerosi, CIBERDEM,

Servei de Medicina Interna Hospital Universitari de Sant Joan,

Institut Investigació Sanitària Pere Virgili

Facultat de Medicina, Universitat Rovira i Virgili, C/ Sant Llorenç, 2143201 Reus, SpainTel: (+34) 977 75 93 69 / (+34) 609 906 991 (mobile) Fax: (+34) 977 75 93 22

E-mail: rosa.sola@urv.cat

and

Mª José Motilva, PhD

Department of Food Technology, XaRTA-UTPV, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida

Avda/Alcalde Rovira Roure 191, 25198 Lleida, Spain.

Tel: +34 973 702817 / Fax: +34 973 702596

E-mail: motilva@tecal.udl.cat

Abbreviations

VOO, virgin olive oil

HT, hydroxytyrosol

SEC, secoiridoids

CVD, cardiovascular diseases

TMT, tandem mass tag

3,4-DHPEA-EDA, 3,4-dihydroxyphenolethanol-elenolic acid di-aldehyde

IPA, Ingenuity Pathway Analysis

PCA, principal component analysis

FC, fold change

VSMC, vascular smooth muscle cells

Abstract

SCOPE: Hydroxytyrosol (HT) is the major phenolic compound in virgin olive oil (VOO) in both free and complex forms (secoiridoids; SEC). Proteomic of tissues related to cardiovascular system such as aorta or heart represents a novel and promising tool to uncover the mechanisms of action of phenolic compounds in healthy animals.

METHODS AND RESULTS: Twelve female Wistar rats were separated into three groups and fed with standard diet and diets supplemented in phenolic compounds (HT and SEC) adjusted to 5 mg/kg/day during 21 days. Proteomic analyses of aorta and heart tissues were performed by nano-liquid chromatography and mass spectrometry. Ingenuity Pathway Analysis was used to generate interaction networks. HT or SEC modulated aorta and heart proteome compared to the control group. The top-scored networks were related to Cardiovascular System Development. The aortic proteins, Rac1, T-kininogen 2, Gja1 and Hsp1a1 that promote proliferation and migration of endothelial cells and occlusion of blood vessels were down-regulated after treatments. In heart, Camk2d and Fkbp1a, related to heart failure, were also positively regulated.

CONCLUSION: Results suggest that free HT reach target cardiovascular tissues inducing changes at proteomic level, which may partially account for the underlying mechanisms involved in the cardiovascular protection of VOO phenols.

Word count: 200

Keywords: hydroxytyrosol, secoiridoids, healthy rats, proteome, cardiovascular disease

Introduction

Hydroxytyrosol (HT) is the major phenolic compound in virgin olive oil (VOO) in either free and complex forms, which are commonly named secoiridoids (SEC) or oleuropein aglycone derivates. HT has shown a wide range of biological functions, such as antioxidant, anticancer and neuroprotective activities, as well as having beneficial effects on the cardiovascular system [1,2]. With a more significant impact, VOO phenolic compounds have been shown to beneficially alter lipid composition, platelet and cellular function as well as reduce the inflammation [3]. These effects have been related with the low rate of cardiovascular disease (CVD) mortality and certain types of cancer in populations residing in the Mediterranean countries [3].

The impact of the diet and dietary components on CVD has been widely recognized in recent years [4,5]. Therefore, prevention through the introduction of lifestyle and proper nutrition habits is now considered a primary strategy for what we call healthy aging. Omics-based studies, including genomics, transcriptomics, proteomics, and metabolomics, have been recognized as powerful analytical tools in cardiovascular research [6]. Specifically, the proteomic approach offers an unbiased way to study changes in protein levels induced by different experimental conditions and a major challenge of proteome research is detecting clinically useful biomarkers of disease, treatment response and aging [7]. Moreover, proteomic approach instead of the analysis of gene expression, focuses on the products that perform the biological function [8].

Despite that proteomics represents a novel and promising tool to uncover the mechanisms of action as a response to diet or nutrients, the actual use of this technique in dietary interventions is still rather limited [9]. Regarding proteomics focused on

cardiovascular tissues, very few studies have been performed up to now investigating the possible protective effect on diseased animals of food bioactive compounds on the protein expression of aortic tissue testing vitamin E and omega-3 fatty acids [10,11] and heart tissue testing resveratrol [12,13]. Other studies analyzing proteome on diseased cardiovascular tissues, such as calcific aortic valve or infarcted myocardium have reflected important changes in protein induced by disease [14]. However, to the best of our knowledge, no study has reported so far the modulation of aorta and heart proteome by bioactive compounds, specifically phenolic compounds, in healthy animals to address future cardiovascular protection. Small animal models have provided insight into the fundamental mechanisms driving early atherosclerosis, but it is increasingly clear that new strategies and research tools are needed to translate these discoveries into improved prevention and treatment of symptomatic atherosclerosis in humans.

The hypothesis of the present work is that VOO phenolic compounds could promote protective effects in cardiovascular system in healthy animals. In an effort to understand the underlying molecular mechanisms of VOO phenols and to identify their potential target protein molecules in cardiovascular tissues, in the present study we performed a proteomic comparative analysis of the aorta and heart tissues of healthy female rats in response to supplemented diet with the equivalent of 5 mg phenol/kg rat weight during 21 days of HT as a pure molecule or its complex occurring forms in VOO through an extract rich in SEC, respectively.

2. Materials and Methods

2.1. Chemicals and reagents

HT was provided by Seprox Biotech (Madrid, Spain), homovanillic acid by Fluka Co. (Steinheim, Switzerland) and catechol from Sigma-Aldrich (St. Louis, MO,

USA). HT-3'-*O*-sulfate was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Methanol and acetonitrile (HPLC-grade) were purchased from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Medford, MA, USA). Tandem mass tag (TMT) 10-plex isobaric reagents were from Thermo Fisher Scientific (San José, CA, USA). Organic solvents were liquid chromatography-mass spectrometry grade from Panreac (Barcelona, Spain). Unless otherwise noted, all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Secoiridoid extract

Ethanolic phenolic extract rich in SEC was obtained from *Arbequina* olive cake by pressurized liquid extraction (ASE 100 Dionex, Sunnyvale, California, USA) based on the method of Suárez *et al* [15],Extraction conditions were: ethanol/water (80:20, v/v) at 80ºC, 60% setting volume and two static cycles of 5 min in each extraction, then, sample was purged with nitrogen (≥ 99.99% purity, Alphagaz, Madrid, Spain). After that, ethanol was rotary evaporated until its elimination (Buchi, New Castle, DE, USA). Aqueous extract was freeze-dried and stored at -80ºC in N_2 atmosphere until use. The extract was mainly composed by dialdehydic form of decarboxymethyl EA linked to HT or 3,4-DHPEA-EDA (85%). It also contained minor proportions of free HT and other secoiridoids providing HT such as the isomer of oleuropein aglycone or 3,4-DHPEA-EA (**Supporting information: Table 1**). In order to calculate the administered dose of 5 mg/kg weight of SEC, only 3,4-DHPEA-EDA was considered as it is the main secoiridoid derivative providing HT.

2.3. Animals and experimental procedure

Twelve female Wistar rats weighted among 300-350 g were obtained from Charles River Laboratories (Barcelona, Spain). They were separated into three groups

with different diets (4 rats in each group): control group (A), HT group (B) and SEC group (C). Animals were housed two per cage in a temperature (21 ± 1ºC) and humidity (55 ± 10%) controlled room with a 12-h light/dark cycle. Food and water were available *ad libitum* in cage and metabolic cages. The animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-Universitat de Lleida, reference 7675).

Supplemented diet preparation was based on follow up the food intake and animal weight during 3 days a week. . Briefly commercial fed pellets (Harlan laboratories, Barcelona, Spain) were crushed andmixed with Milli-Q water containing the equivalent of 5 mg of HT or SEC/kg rat weight in average of daily consumption of each rat and were then freeze-dried.

After 21 days of treatment rats were sacrificed by intracardiac puncture after isoflurane anaesthesia (IsoFlo, Veterinarian Esteve, Bologna, Italy). After blood collection, rats were perfused by isotonic solution of NaCl 0.9% to remove the remaining blood irrigating tissues and heart and aorta were excised from the rats. All samples were stored at – 80ºC until analysis.

2.4. Heart tissue sample pre-treatment and phenolic chromatographic analysis

In order to study the disposition of phenolic compounds on heart tissue after HT or SEC treatments, the free HT and its biological metabolites were determined. Aorta was not analyzed as it was entirely used for proteomic analysis. Prior to the chromatographic analysis, heart was freeze-dried and homogeneus samples were sequentially pre-treated with a combination of liquid-solid extraction combined with micro solid phase extraction as previously described [16]. Phenolic compounds were then analyzed by Acquity Ultra-Performance™ liquid chromatography coupled to

tandem MS as the detector system from Waters (Milford, MA, USA), as reported in our previous study [16].

2.5. Proteomic sample preparation and quantitative analysis of the aorta and heart tissues

A quantitative proteomic study in aorta and heart rat tissues was performed using TMT isobaric tag labelling, off-gel fractionation and mass spectrometry based on nanoLC-Orbitrap technology was performed (**Figure 1**). The different analytical steps are explained in detail in **supporting information: Materials and Methods**.

2.6. Clustering and pathway analysis

Various bioinformatics tools were employed for the biological interpretation of the results. Proteins were referred to by their gene encode symbol. Each protein identifier (Swiss-Prot ID) was mapped to its corresponding protein object in the Wiki Pathways, KEGG, Reactome and BioCyte data bases.

Ingenuity Pathway analysis (IPA; Ingenuity System Inc., Redwook, CA, USA, www.ingenuity.com) was used to analyze canonical pathways and protein networks involving the differentially expressed proteins for biological interpretation. Significance levels were assessed with Fisher's exact tests (p<0.01). The differentially expressed proteins were overlaid with IPA-curated canonical pathways to explore possible metabolic and cell signaling pathways that were over- or under-represented by the experimentally determined genes. Specifically, we conducted two analysis: a) the common proteins up- or down-regulated in the same direction after both treatments (HT and SEC) in aorta or heart tissue compared to control group, to study the potential common HT and SEC mechanisms of action, and b) the whole dataset of proteins differentially expressed after HT or SEC treatments in aorta or heart tissue compared to

control group in order to acquire a global vision and focused on cardiovascular system (**Figure 2**).

In addition, possible connections between mapped genes were evaluated and graphical networks were algorithmically generated. Nodes representing genes and gene products were linked by biological relationships. Networks were ranked by a score that defines the probability of a collection of nodes being equal to or greater than the number in a network achieved by chance alone.

2.7. Statistical analysis

The statistical analysis to find the significant protein changes between conditions included in the present studies was done by Mass Profiler Professional software v. 13.0 from Agilent Technologies (Santa Clara, CA, USA).

The statistical analysis was the same for aortic and heart tissue. The study comprises three different groups named as control, HT and SEC. A 1-Way ANOVA statistical test was applied with a *p*-value correction of Benjamin-Hochberg false discovery rate for multiple comparisons using the quantified proteins that appears in more than 66% of samples. Moreover multivariate statistical technique based on principal components analysis (PCA) was applied. There are 3 replicates for control condition (one is used for normalization and is not considered for statistical analysis) and 4 replicates for each treatment condition.

3. Results

3.1. Disposition of HT and its metabolites in heart tissue

As shown in **Table 1**, after diet supplementation with 5 mg of HT or SEC/kg weight/day during 21 days, free HT was detected in heart tissue mainly in its free form and in a minor proportion as phase II metabolite HT-3-*O*-sulfate, which only appeared

after HT treatment. When comparing both treatments, the free form of HT presented significant higher concentrations after SEC diet supplementation compared to HT.

The free HT detected in the heart tissue of the control group could be related to the endogenous origin of this compound from dopamine metabolism previously described [17].

3.2. Tissue proteome modulation by HT and SEC

3.2.1. Aorta

After performing the proteome analysis of aorta tissue samples, we reached to identify and quantify 1247 proteins. The comparative analysis revealed that from the identified proteins, 115 significantly differed after HT and SEC treatments compared to control group (p<0.05). All these up- or downregulated proteins with their corresponding fold change (FC) values compared to the control group are listed in **Table 2** and they have been also classified in a Venn diagram in **Figure 2A**. The corresponding information for all identified proteins in aorta is available in **supporting information: Table 2.**

The comparative analysis between HT treatment and control group in aorta tissue revealed an up-regulation of 26 proteins ranging from 1.0 to 1.3 FC and a down-regulation of 89 proteins from -1.0 to -1.7 of FC. When comparing SEC treatment with control group, we observed an up-regulation of 34 proteins ranging from 1.2 to 16.3 of FC and a down-regulation of 81 proteins from -1.2 to -18.8 of FC. As shown in the Venn diagram (**Figure 2A)** most of these proteins were significantly modulated in the same direction after the two treatments (HT or SEC) compared to control group, and in most of the proteins FC values were strengthened after SEC diet supplementation compared to HT (**Table 2**). The PCA of aortic tissue samples after the statistical test showed that only a slightly differentiation between control group and HT group is

observed but both are significantly different compared to SEC (**Supporting information: Figure 1A**), which is in accordance with the higher FC values of SEC.

3.2.2. Heart

In heart tissue we identified and quantified 1124 proteins and 34 proteins that significantly differed after HT and SEC treatments compared to control group (p<0.05). All the up- or downregulated proteins with their corresponding FC values compared to the control group are listed in **Table 3** and have been classified in a Venn diagram shown in **Figure 2B**. The corresponding information for all the identified proteins in heart is available in **supporting information: Table 3**.

Comparative between HT treatment and control group revealed an up-regulation of 12 proteins ranging from 1.0 to 1.4 of FC and a down-regulation of 22 proteins ranging from -1.0 to -1.4 of FC. Comparing SEC treatment to control group, we observed an up-regulation of 22 proteins ranging from 1.0 to 3.4 of FC and a down-regulation of 12 proteins ranging from -1.1 to -1.5 of FC. Comparing SEC treatment to HT treatment revealed that 23 proteins were up-regulated (from 1.0 to 3.3 of FC) and 11 were down-regulated (from -1.0 to -2.0 of FC). As in aorta, some of the proteins were differentially expressed in the same direction after the two treatments (HT or SEC) compared to control group (**Figure 2B**), but in heart, most of the proteins were modulated in opposite directions depending on the treatment. The PCA analysis showed a clear differentiation between the three groups (control, SEC and HT) in heart tissue (**Supporting information: Figure 1B**).

Among all the differentially expressed proteins, only two proteins, Actn4 and Rpl8, were found to be significantly modulated in both aorta and heart tissues.

3.3. Pathway analysis of common proteins modulated by HT and SEC

Following the pathway analysis strategy "a" described in section 2.6, we analyzed in IPA the common proteins that were differentially up- or downregulated in the same direction in response to both the HT and SEC consumption in order to investigate common mechanisms after both treatments. Results regarding the top-scored canonical pathways, molecular and cellular functions, diseases and disorders and potential upstream regulators are presented in detail in **Supporting information: Results**.

3.4. Global proteome changes after HT or SEC and its relation with Cardiovascular System

Following the analysis strategy "b" the whole dataset of significantly expressed proteins after HT and SEC treatments in aorta (115 proteins) or heart (34 proteins) tissue compared to control group was analyzed in IPA with special focus on Cardiovascular System functions.

3.4.1 Aorta tissue

When the complete dataset of proteins differentially expressed in aorta was analyzed, the top network found by IPA was "Cardiovascular System Development" (score = 11). 11 proteins of the 115 regulated proteins in aorta were part of this network. The graphical representation of the network is shown in **Figure 3**, in which the modulated proteins (located in the cell compartments) have been highlighted in color and indicated when HT and SEC differently modulated a protein (up or down-regulated).

We observed a significant increase of Hk2, Fabp5, Ldh proteins and a decrease of Akr1b1, Capn2, Gja1, Rac1, Ilk, Vcl and Kng1/Kng1l1 proteins after both treatments. Vim protein was up-regulated by HT treatment and down-regulated after SEC treatment. Some of these proteins (Kng1, Hk2, Gja1 and Rac1) were found by IPA to have a

strong relation with specific cardiovascular system functions, which are listed in **Table 4**. These proteins were mainly related to cardiac functions in this tissue such as occlusion of blood vessel and proliferation of endothelial cells.

Other proteins implicated in the network and connected with the proteins detected in our study were: NF-κB, Hspb1, Prkce, Pten, Mapk14, Prkca, Nos2, Erk1/2, Pka, Akt, Pdpk1, Pak2, Pxn, Rhoa, Nos3, Casp3, Pik3r1, Dock7, Cav3, Agtr2, Slc2a4, Igf1r, Cav1 and Ctgf. As shown NF-κB complex and Akt appeared to be key proteins in the network.

3.4.2. Heart tissue

In the case of heart tissue, the top network found by IPA was "Cardiovascular System Development and Cancer" (score = 57). 21 proteins of the 34 regulated proteins in heart were part of this network, which is represented in **Figure 4**.

The up-regulated proteins after HT and SEC treatments implicated in this network were Npm1, Fkbp1a, Tuba4a, Arhgdia, Msn and Alpha tubulin proteins and the down-regulated were Por, Dlat, Dld, Cys, Cytochrome C and Magi 2 proteins (**Figure 4**). Some proteins, such as Hmgb1, Hbb, Actn4, Psma6, Dnm1l, Map4 and Clic4, were down-regulated by HT treatment and up-regulated in response to SEC treatment. On the other hand, Samm50, Glrx3, Prep and Camk2d were up-regulated by HT treatment and down-regulated after SEC treatment. As in aorta, some of these proteins had a strong relation with specific cardiovascular system functions, which are listed in **Table 4**. Some of these proteins such as Camk2d, Fkbp1a and Tuba4a, were related to cardiac functions and other proteins such as Dlat, Dld and Por were related to lipid metabolism. Other proteins implicated in the network were Ppil4, Nf-kB, estrogen receptor, Hsp90, Calcineurin proteins, Caspase, Akt, P38 Mapk, Pka, Cd3 and N-methyl-R-salsolinol. NF-κB complex also resulted in a pivotal position as in aorta network.

4. Discussion

Under the hypothesis that VOO phenolic compounds could promote benefits in cardiovascular system by inducing changes in the heart and aorta protein levels, in the present study we performed a proteomic analysis of the aorta and heart tissues of healthy rats in response to a diet supplemented with HT to investigate the underlying molecular mechanism of HT and to identify potential target proteins in cardiovascular tissues. HT was administrated either as a pure molecule or through its complex occurring forms in VOO (SEC) at the same daily dose (5 mg/kg rat weight) in order to investigate possible differences in the molecular mechanisms depending on the chemical structure administered.

4.1. Aorta and heart proteome modulation by HT and SEC

Proteomic analysis revealed that the heart and aorta proteome significantly changed after the administration of VOO phenolics compared to the control group, observing a clearer differentiation when diet was supplemented with SEC (**Supporting information: Figure 1**). As expected, most of the proteins were similarly modulated after SEC and HT and FC values did not exceed in most cases 2.5-fold. The relatively low FC after the supplemented diets could be attributed to the healthy status of the animals. Previous studies have focused on the proteomic profiling of aorta diseased tissues in order to gain knowledge of the molecular events underlying pathological processes such as the atherosclerotic lesions [18]. However, based on the general consensus that VOO phenolic compounds have a protective role in the cardiovascular system [1], in this study we present the proteomic technique applied to healthy cardiovascular tissues as a novel approach to gain knowledge in the possible prevention mechanisms of these bioactive compounds.

There are scarce data in the bibliography regarding the effect of olive phenols on the tissue proteome modulation and so far no studies have been performed with heart and aorta tissues. Only some studies performed in injured liver of rat or mice and the hepatic proteome analysis revealed that VOO phenolic compounds could have benefits against chronic liver injury and steatosis, which was attributed to the modulation of proteins related to antioxidant mechanisms [19–21]. In other studies, the effect of VOO on the plasma proteome during aging in rats was analyzed [22] and also the impact on the HDL protein cargo of the intake of VOO and two other phenol-enriched VOO in hypercholesterolemic subjects [23]. In both cases, a modulation of proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation was observed.

When comparing HT and SEC, diet supplementation with SEC leaded to higher FC values. These differences could be attributed to the higher concentration of free HT detected in heart tissue after SEC diet (**Table 1**). In our previous work it was demonstrated that despite that HT and SEC were administered at the same dose (5 mg/kg rat weight), the urinary recovery of HT metabolites was higher with SEC, indicating that the bioavailability of HT was more effective with the intake of the more complex structure as SEC (*Lopez de las Hazas 2016; J Func Foods, submitted)*. Consequently, higher amounts of HT and HT metabolites were able to reach the target tissue (heart) and could have exerted a greater modulation of the proteome. These results highlight that the complementary information regarding the bioavailability and the tissue disposition of the phenolic metabolites is critical to understand and determine the bioactivity exerted in that tissue. Our results demonstrate that phenolic compounds were effectively absorbed and could reach target tissues such as heart. In accordance, in a previous study performed by our group demonstrated that the free HT at physiological

concentrations and in a dose-dependent manner, could exert significant effects reducing adhesion molecules (vascular adhesion molecule-1, intracellular adhesion molecule-1, E-selectin and P-selectin) and in a chemokine (monocyte chemoattractant protein-1) in human aortic endothelial cells stimulated by tumor necrosis factor alpha-1 [24].

4.2. Pathway analysis of common proteins modulated by HT and SEC

In a first step, we analyzed in IPA the common proteins that were differentially expressed in the same direction after HT and SEC supplementation. From these results we highlight the potential upstream regulators that appeared to be implicated, which allowed us to identify the molecules upstream of the proteins that potentially explain the observed expression changes.

In the case of aorta, Myc, Myocd, Pias1, Yap1 and Srf appeared to be potentially implicated in the modulated pathways. All these proteins have been shown to play a role in proliferative vascular disorders in vascular smooth muscle cells (VSMC) [25]. Specifically, Yap1, Myc and Myocd have been related in the modulation of VSMC phenotype in response to the environmental stimuli through a process characterized by an increased proliferation and migration [26]. Phenotypic switch of VSMC is one of the major cellular events underlying many VSMC-related pathological conditions, such as atherosclerosis.

In heart tissue, the top upstream regulators that appeared to be potentially implicated were E2f4 and PPAR-γ. The transcription factor E2f4 has been shown to act as an activator of cardiomyocyte proliferation, a required regulator for cardiac regeneration [27]. PPAR-γ has been well recognized to be a central player participating in various biological responses, including lipid metabolism, inflammation, and cell proliferation, the underlying pathological processes of metabolic diseases and cancer [28]. So, the potentially implication of these upstream regulators in aorta and heart

tissues in response to olive phenolic compounds provides insight into the molecular mechanisms underlying their protective action on cardiovascular alterations.

4.3. Global network analysis after HT or SEC and its relation with Cardiovascular System

The network analysis based on the whole expressed proteins after HT and SEC diets demonstrated that the top-scored networks were related to Cardiovascular System Development in both tissues (**Figures 3 and 4**), and specifically, some of the modulated proteins appeared to be involved in particular cardiac functions and lipid metabolism (**Table 4**). In the case of aorta (**Figure 3**), the proteins involved in the top-scored network were mainly related to several cardiac functions. One of the down-regulated proteins was T-kininogen 2 (-1,2-fold and -2,7-fold for HT and SEC respectively), involved in the occlusion of blood vessel and proliferation of endothelial cells. Kininogens are multifunctional proteins that act as precursor of kinins, small vasoactive peptides that promote endothelial cell proliferation through kinin receptors and its plasma levels have been related to inflammatory and aging processes in rats and humans [29]. In accordance with our results, T-kininogen precursor was significantly decreased after the intake of VOO compared with sunflower oil in rats [22]. Therefore, the decrease of T-kininogen 2 after intake of HT and SEC suggests that HT could contribute to the decline of inflammatory processes. Together with T-kininogen 2, Gap junction alpha-1 protein (Gja1), was also related to proliferation of endothelial cells, as well as with cardiogenesis and vasoconstriction of blood vessel and, in the same line, it was down-regulated after HT (-1,0-fold) and SEC (-1.7-fold) treatments.

Another relevant protein involved in the top-scored Cardiovascular System network in aorta was Rac1 (ras-related C3 botulinum toxin substrate 1), which was

significantly down-regulated after HT (-1,0-fold) and SEC (-1,4-fold) diets and appeared to be implicated in the migration of endothelial cells and heart rate functions (**Table 4**). Rac1 is a small GTPase essential for the assembly and activation of NADPH oxidase. Several molecular and cellular studies have reported the involvement of Rac1 in different cardiovascular pathologies, such as vascular smooth muscle proliferation, atherosclerosis and endothelial dysfunction [30]. Also an increased activation of NADPH oxidase by Rac1 has also been reported in animals and humans after myocardial infarction and heart failure [30]. Due to all these findings, Rac1 has emerged as a new pharmacological target for the treatment of CVD [31]. Thus, our results suggest that its down-regulation after HT and SEC consumption could imply a cardio-protection effect.

Hexokinase-2 (HK2) also appeared to be one of the differentially expressed proteins (1,3-fold for HT and 2,7-fold for SEC) related to the cardiovascular network in aorta and playing an important role in cell viability of cardiomyocytes and survival of ventricular myocytes (**Table 4**). HKs are multifunctional proteins that orchestrate metabolic, antioxidant and direct anti-cell death effects [32]. HK2 specifically has a role in shuttling glucose-6-phosphate to glycolysis and oxidative phosphorylation when bound to mitochondria [33]. Genetic reduction of HK2 levels in heterozygous HK2 knock-out mice increased susceptibility to ischemia/reperfusion injury [34] indicating its cardioprotective role. In this sense, HK2 also increased the cell viability of cultured ventricular myocytes [35]. Therefore, the overexpression of HK2 after HT and SEC administration suggests that HK2 could be an important protein target for HT. Hspe1 (up-regulated by HT 1,1-fold and SEC 2,5-fold) was also related to cardiac necrosis/cell death observed by previous studies in which its up-regulation decreased apoptosis of cardiac myocytes from newborn rat [36].

In the same line of cardio-protection, heat shock 70 kDa protein (Hspa1a) was down-regulated after both treatments (-1.0-fold for HT and -2.3-fold for SEC). Hspa1a plays a multiple role in cellular homeostasis and its level increases rapidly in response to various types of severe stress. In fact, it has been related to the development of atherosclerosis [37], and also seems to be a clinically useful biomarker for prediction of mortality in heart failure patients [38]. Apart from the implication of Hsp1a1 in the cardiovascular system, its overexpression has been linked to the development of some cancers, such as hepatocellular carcinoma, gastric cancers, colon cancers, breast cancers, and lung cancers, which led to its use as a prognostic marker for these cancers [39]. Circulating Hspa1a has been also recently stablished as a clinical marker of rheumatoid arthritis, used for its diagnosis and monitoring the disease activity [40]. Moreover, the over-expression of Annexin 2 (Anxa2), which was down-regulated after both diets (-1,0-fold for HT and -1,4-fold for SEC), has been defined as a prognostic marker in certain cancers such as cholangiocarcinoma [41]. These results together with all the presented evidence, suggest that olive phenols not only might be able to modulate proteins related to the prevention of CVD, but could also exert a protector effect against cancer modulating some cancer-relevant proteins.

The network analysis in heart tissue revealed that the significantly expressed proteins were implicated in cardiac functions but also in the lipid metabolism. Fkbp1a (peptidyl-prolyl cis-trans isomerase), which appeared to be related to heart failure, hypertrophy of left ventricle, congestive heart failure and systolic dysfunction, was up-regulated 1.2-fold and 1,7-fold by HT and SEC, respectively. Previous studies show that mutant mice deficient in Fkbp1a develop multiple abnormalities in cardiac structure, including lack of compaction and thin ventricular walls [42], suggesting that its up-regulation could imply a positive effect preventing all these anomalies. In the same line,

Calcium/calmodulin-dependent protein kinase type II subunit delta (Camk2d) was also modulated and it has been defined as a determinant of clinically important heart disease phenotypes, and it has been suggested that its inhibition can be a highly selective approach for targeting adverse myocardial hypertrophy, dilation and dysfunction, in individuals with myocardial infarction [43].

Other down-regulated proteins in heart tissue (Por, Dlat and Dld) appeared to be related to lipid metabolism functions such as synthesis of acetyl-CoA, fatty acid metabolism and synthesis of lipids. In particular, NADPH-cytrochome p450 reductase (Por) is an enzyme that is required for electron transfer to cytochrome P450 enzymes and plays a major role in regulating lipid homeostasis. On the other hand, Dlat and Dld play a role in energy metabolism, specifically in the pyruvate metabolism.

Another related protein that appeared to be down-regulated was Cythocrome c (Cycs), a component of the electron transport chain in mitochondria involved in initiation of apoptosis. Cycs release in cardiomyocytes has been attributed to many mechanisms including ROS generation, cardiolipin peroxidation, and Ca^{2+} overload in the mitochondria and therefore, if the release of Cytc could be inhibited, apoptosis could be prevented, slowing the disease progression or limiting neurologic damage after trauma [44].

Interpreting the findings of the present proteomic study is mitigated by a limited understanding of the individual role of implicated proteins within the tissue. The differentially expressed proteins have been localized to specific signaling pathways or networks but the interplay between pathways is complex and incompletely understood. Nevertheless, the findings of the present study clearly revealed that HT and SEC may

play a pivotal role in the Cardiovascular System through the modulation of several proteins.

In conclusion, proteomic analysis revealed that the heart and aorta proteome significantly changed after the diet supplementation with HT and SEC compared to the control group. As expected, most of the proteins were similarly modulated after HT and SEC, and FC values did not exceed in most cases 2.5 fold, which was attributed to the healthy status of the animals. The network analysis involving the differentially expressed proteins revealed that the top-scored networks were related to cardiovascular system in both aorta and heart tissues. Specifically, our results demonstrated that HT and SEC are able to positively regulate the expression of relevant proteins (Rac1, T-kininogen 2, Gja1 and Hsp1a1) in aorta tissue related to atherosclerotic processes such as proliferation and migration of endothelial cells and occlusion of blood vessels. In heart, the more relevant modulated proteins (Camk2d and Fkbp1a) were related to cardiac functions such as heart failure.

Moreover, other prognostic markers for some cancers, Hsp1a1 and Anxa2, were modulated after HT and SEC treatments, suggesting that olive phenols could also exert a protector effect against cancer.

Diet supplementation with SEC demonstrated higher FC values which was attributed to the higher concentration of HT detected in heart tissue as consequence of the more effective bioavailability and heart tissue disposition. These results suggest that SEC, the main phenolics present in VOO, could have a higher cardio-protective effect than free HT.

In this study we present the proteomic technique applied to healthy cardiovascular tissues as a novel approach to gain knowledge in the possible root causes

for the VOO phenol protective effects in cardiovascular system. Nevertheless further studies are needed to asses SEC or HT supplementation in animals with a cardiovascular pathology.

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The authors have declared no conflict of interest.

6. Figure legends

Figure 1. Experimental workflow chart.

Figure 2. Venn diagram of aorta and heart tissue. Venn diagram showing intersections of proteins differentially expressed in aorta **A**) or heart **B**) tissue of healthy rats comparing HT *versus* control and SEC *versus* control group. Red color indicates up-regulated proteins and green color indicates down-regulated proteins.

Figure 3. Aorta Cardiovascular System Network after HT or SEC treatments. Interaction between the proteins differentially expressed in aorta after HT or SEC treatments and other important proteins related to the same network. HT or SEC are represented in red or green color if the protein is up- or down-regulated respectively after HT or SEC treatments.

Figure 4. Heart Cardiovascular System Network after HT or SEC treatments. Interaction between the proteins differentially expressed in heart after HT or SEC treatments and other important proteins related to the same network. HT or SEC are

represented in red or green color if the protein is up- or down-regulated respectively after HT or SEC treatments.

Supporting information: Figure 1. Principal component analysis (PCA) for differentially expressed proteins of **A**) aorta tissue or **B**) heart tissue samples of control (■), HT (▲) or SEC (●) group.

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Table 1. Concentration (nmols/g fresh tissue) of free hydroxytyrosol and the main metabolite, hydroxytyrosol-3-*O*-sulfate, detected in heart tissue after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroytyrosol (HT) or secoiridois (SEC).

	Control (n=4)	SEC (n=4)	HT (n=4)
Free hydroxytyrosol	5.78 (0.02)[a]	19.6 (1.56)[c]	8.75 (0.82)[b]
Hydroxytyrosol-3-*O*-sulfate	n.d.	n.d.	0.52 (0.18)[a]

n.d.: non detectable

[a. b. c] indicates significant differences among treatments in the same row at the 95.0% level of confidence. Results are expressed as mean (standard deviation; SD)

Table 2. 115 significant proteins differentially expressed in aortic tissue of healthy rats after HT or SEC treatments.

Swiss-Prot code	Gene symbol	Protein Name	MW (kDa)	*p*	FC (HT *versus* Control group)	FC (SEC *versus* Control group)	FC (SEC *versus* HT group)
Q9QZA2	Pdcd6ip	Programmed cell death 6-interacting protein	96.6	1.4E-05	-1.1	-2.0	-1.7
P62850	Rps24	40S ribosomal protein S24	15.4	2.4E-05	-1.1	-2.2	-2.1
E9PT87	Mylk3	Myosin light chain kinase 3	85.5	3.1E-05	-1.0	1.9	1.9
P62074	Timm10	Mitochondrial import inner membrane translocase subunit Tim10	10.3	3.3E-05	1.0	3.1	3.0
P60868	Rps20	40S ribosomal protein S20	13.4	3.3E-05	-1.1	3.3	3.5
P62282	Rps11	40S ribosomal protein S11	18.4	3.4E-05	-1.1	-1.6	-1.5
P10860	Glud1	Glutamate dehydrogenase 1. mitochondrial	61.4	3.9E-05	-1.1	-1.5	-1.4
P06907	Mpz	Myelin protein P0	27.6	3.9E-05	-1.2	15.9	19.8
P62914	Rpl11	60S ribosomal protein L11	20.2	5.0E-05	-1.0	1.9	1.9
Q99MC0	Ppp1r14a	Protein phosphatase 1 regulatory subunit 14A	16.7	7.7E-05	1.0	-2.2	-2.2
Q9EPF2	Mcam	Cell surface glycoprotein MUC18	71.3	8.3E-05	1.1	-1.7	-1.9
P17077	Rpl9	60S ribosomal protein L9	21.9	9.5E-05	-1.1	-1.7	-1.5
Q5I0E7	Tmed9	Transmembrane emp24 domain-containing protein 9	27.0	1.1E-04	-1.1	1.8	2.0
P21531	Rpl3	60S ribosomal protein L3	46.1	1.2E-04	-1.0	-1.7	-1.7
P62083	Rps7	40S ribosomal protein S7	22.1	1.3E-04	-1.2	-1.8	-1.4
Q6AY20	M6pr	Cation-dependent mannose-6-phosphate receptor	31.1	1.4E-04	-1.0	-1.4	-1.3
P62919	Rpl8	60S ribosomal protein L8	28.0	1.5E-04	1.1	1.7	1.6
P27274	Cd59	CD59 glycoprotein	13.8	1.5E-04	1.1	1.5	1.4
Q9Z270	Vapa	Vesicle-associated membrane protein-associated protein A	27.8	1.6E-04	1.0	1.6	1.6
P04904	Gsta3	Glutathione S-transferase alpha-3	25.3	2.0E-04	-1.1	-1.7	-1.6
Q4KMA2	Rad23b	UV excision repair protein RAD23 homolog B	43.5	2.1E-04	-1.1	-1.4	-1.3
P17074	Rps19	40S ribosomal protein S19	16.1	2.3E-04	-1.1	1.4	1.5
Q9QWN8	Sptbn2	Spectrin beta chain. brain 2	270.9	2.3E-04	-1.0	1.4	1.5
F1LMY4	Ryr1	Ryanodine receptor 1	565.1	2.4E-04	-1.4	1.4	2.0
P05539	Col2a1	Collagen alpha-1(II) chain	134.5	2.8E-04	-1.7	16.3	28.4
P18445	Rpl27a	60S ribosomal protein L27a	16.6	3.1E-04	-1.0	-1.3	-1.3
P08010	Gstm2	Glutathione S-transferase Mu 2	25.7	3.4E-04	-1.1	-2.1	-1.9
P50609	Fmod	Fibromodulin	43.2	3.5E-04	1.0	-3.5	-3.6
B5DFC9	Nid2	Nidogen-2	152.9	3.6E-04	-1.1	1.5	1.7
Q9EPH8	Pabpc1	Polyadenylate-binding protein 1	70.7	3.8E-04	1.1	-1.2	-1.3
P05545	Serpina3k	Serine protease inhibitor A3K	46.5	4.1E-04	-1.6	1.3	2.1
Q5XFX0	Tagln2	Transgelin-2	22.4	5.1E-04	-1.2	-1.9	-1.6
A2RUV9	Aebp1	Adipocyte enhancer-binding protein 1	128.0	5.5E-04	-1.3	-2.3	-1.7
B2RZ78	Vps29	Vacuolar protein sorting-associated protein 29	20.5	5.6E-04	-1.1	-1.5	-1.4
P23928	Cryab	Alpha-crystallin B chain	20.1	7.0E-04	1.3	-5.6	-7.0
P04692	Tpm1	Tropomyosin alpha-1 chain	32.7	7.4E-04	1.1	-1.9	-2.2
P53812	Pitpnb	Phosphatidylinositol transfer protein beta isoform	31.4	7.5E-04	-1.0	-1.4	-1.4
A4L9P7	Pds5a	Sister chromatid cohesion protein PDS5 homolog A	150.2	8.2E-04	-1.3	1.7	2.2
O08619	F13a1	Coagulation factor XIII A chain	82.6	8.4E-04	-1.0	-1.4	-1.4
P61206	Arf3	ADP-ribosylation factor 3	20.6	8.9E-04	-1.1	-1.5	-1.4

Q63357	Myo1d	Unconventional myosin-Id	116.0	8.9E-04	-1.1	-2.1	-1.8
P16257	Tspo	Translocator protein	18.9	9.9E-04	-1.0	1.6	1.6
P70470	Lypla1	Acyl-protein thioesterase 1	24.7	1.1E-03	-1.1	1.4	1.5
Q4V8C3	Eml1	Echinoderm microtubule-associated protein-like 1	89.7	1.1E-03	-1.1	-1.7	-1.6
P24050	Rps5	40S ribosomal protein S5	22.9	1.2E-03	-1.0	-1.2	-1.1
P0C5E3	Palld	Palladin (Fragment)	66.7	1.4E-03	-1.0	-1.5	-1.5
Q08290	Cnn1	Calponin-1	33.3	1.4E-03	1.1	-3.8	-4.3
Q07439	Hspa1a	Heat shock 70 kDa protein 1A/1B	70.1	1.4E-03	-1.0	-2.3	-2.2
P40307	Psmb2	Proteasome subunit beta type-2	22.9	1.5E-03	-1.1	-1.4	-1.3
P05544	Serpina3l	Serine protease inhibitor A3L	46.2	1.5E-03	-1.6	1.2	1.9
P63039	Hspd1	60 kDa heat shock protein. mitochondrial	60.9	1.6E-03	-1.1	1.5	1.7
P08932	N/A	T-kininogen 2	47.7	1.7E-03	-1.2	-2.7	-2.3
Q9QXQ0	Actn4	Alpha-actinin-4	104.8	1.8E-03	-1.1	-1.7	-1.6
P58775	Tpm2	Tropomyosin beta chain	32.8	1.8E-03	-1.1	-2.1	-1.9
D3Z9R8	Mp6	6.8 kDa mitochondrial proteolipid	6.9	1.8E-03	-1.0	-4.1	-4.0
P24054	Sparcl1	SPARC-like protein 1	70.6	1.8E-03	-1.4	-2.8	-2.0
P08082	Cltb	Clathrin light chain B	25.1	2.0E-03	-1.1	-1.7	-1.6
Q9Z1P2	Actn1	Alpha-actinin-1	102.9	2.1E-03	-1.1	-2.5	-2.2
Q01129	Dcn	Decorin	39.8	2.1E-03	-1.1	-1.4	-1.3
Q9R063	Prdx5	Peroxiredoxin-5. mitochondrial	22.2	2.2E-03	1.1	1.6	1.5
P51886	Lum	Lumican	38.3	2.3E-03	-1.1	-1.6	-1.4
Q63862	Myh11	Myosin-11 (Fragments)	152.4	2.5E-03	-1.1	-1.6	-1.5
P61983	Ywhag	14-3-3 protein gamma	28.3	2.6E-03	1.0	1.5	1.5
P14046	A1i3	Alpha-1-inhibitor 3	163.7	2.7E-03	-1.2	-1.9	-1.6
P31232	Tagln	Transgelin	22.6	2.8E-03	-1.1	-2.0	-1.8
Q62745	Cd81	CD81 antigen	25.9	2.9E-03	-1.1	-2.0	-1.8
Q68FT1	Coq9	Ubiquinone biosynthesis protein COQ9. mitochondrial	35.1	3.0E-03	1.1	2.1	1.9
P16975	Sparc	SPARC	34.3	3.1E-03	-1.1	-3.0	-2.8
P41123	Rpl13	60S ribosomal protein L13	24.3	3.1E-03	-1.1	-2.0	-1.9
Q923W4	Hdgfrp3	Hepatoma-derived growth factor-related protein 3	22.4	3.1E-03	-1.2	-1.5	-1.3
Q9EST6	Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	31.0	3.2E-03	1.0	-1.4	-1.4
P08050	Gja1	Gap junction alpha-1 protein	43.0	3.5E-03	-1.0	-1.7	-1.7
P62278	Rps13	40S ribosomal protein S13	17.2	3.7E-03	1.1	-1.3	-1.3
Q4QQT4	Ppp2r1b	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform	66.0	3.7E-03	1.2	1.6	1.3
Q7M0E3	Dstn	Destrin	18.5	3.8E-03	-1.1	-2.1	-1.8
P10111	Ppia	Peptidyl-prolyl cis-trans isomerase A		3.8E-03	-1.1	-1.5	-1.3
P13635	Cp	Ceruloplasmin	120.8	3.9E-03	-1.4	-1.6	-1.2
D3Z8E6	Camsap1	Calmodulin-regulated spectrin-associated protein 1	178.4	4.1E-03	-1.1	-1.6	-1.4
P85972	Vcl	Vinculin	116.5	4.3E-03	-1.1	-1.6	-1.4
Q4V9H5	Phf20l1	PHD finger protein 20-like protein 1	114.0	4.5E-03	1.0	2.0	1.9
Q4V7C7	Actr3	Actin-related protein 3	47.3	4.6E-03	-1.2	-1.5	-1.3
Q8VIF7	Selenbp1	Selenium-binding protein 1	52.5	4.7E-03	-1.1	-1.4	-1.3
Q62812	Myh9	Myosin-9	226.2	4.9E-03	-1.1	-1.5	-1.4
Q0ZHH6	Atl3	Atlastin-3	60.5	5.0E-03	-1.1	-1.4	-1.2
Q99J82	Ilk	Integrin-linked protein kinase	51.3	5.2E-03	-1.1	-1.8	-1.6
Q62908	Csrp2	Cysteine and glycine-rich protein 2	20.9	5.2E-03	-1.2	-2.2	-1.9

P55053	Fabp5	Fatty acid-binding protein. epidermal	15.0	5.3E-03	1.0	2.5	2.4
Q63610	Tpm3	Tropomyosin alpha-3 chain	29.0	5.3E-03	1.1	-1.7	-1.8
P11884	Aldh2	Aldehyde dehydrogenase. mitochondrial	56.5	5.3E-03	-1.1	-1.6	-1.4
Q9QYP2	Celsr2	Cadherin EGF LAG seven-pass G-type receptor 2 (Fragment)	233.3	5.4E-03	-1.2	-3.0	-2.5
Q9JHY2	Sfxn3	Sideroflexin-3	35.4	5.6E-03	-1.2	-1.9	-1.6
B2GUZ5	Capza1	F-actin-capping protein subunit alpha-1	32.9	5.8E-03	-1.1	-1.6	-1.5
B5DEH2	Erlin2	Erlin-2	37.7	6.1E-03	-1.1	1.4	1.4
P48037	Anxa6	Annexin A6	75.7	6.4E-03	-1.0	-1.3	-1.2
Q6RUV5	Rac1	Ras-related C3 botulinum toxin substrate 1	21.4	6.5E-03	-1.0	-1.4	-1.3
Q9Z1B2	Gstm5	Glutathione S-transferase Mu 5	26.6	6.6E-03	-1.1	-1.5	-1.3
P54311	Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	37.4	6.6E-03	-1.1	-1.5	-1.3
P45592	Cfl1	Cofilin-1	18.5	6.6E-03	-1.1	-1.6	-1.5
P62271	Rps18	40S ribosomal protein S18	17.7	6.6E-03	-1.0	-1.7	-1.7
Q6IRK9	Pgcp	Plasma glutamate carboxypeptidase	52.0	6.8E-03	-1.0	1.8	1.8
P04642	Ldha	L-lactate dehydrogenase A chain	36.4	7.0E-03	1.1	1.9	1.7
P41562	Idh1	Isocitrate dehydrogenase [NADP] cytoplasmic	46.7	7.0E-03	-1.1	-1.3	-1.2
P27881	Hk2	Hexokinase-2	102.5	7.1E-03	1.3	2.7	2.0
P50503	St13	Hsc70-interacting protein	41.3	7.2E-03	1.0	-1.8	-1.8
P62828	Ran	GTP-binding nuclear protein Ran	24.4	7.3E-03	-1.0	1.3	1.3
P31000	Vim	Vimentin	53.7	7.4E-03	1.0	-1.9	-1.9
Q62667	Mvp	Major vault protein	95.7	7.4E-03	-1.0	1.4	1.4
P85973	Pnp	Purine nucleoside phosphorylase	32.3	7.6E-03	-1.2	-1.5	-1.3
Q03626	Mug1	Murinoglobulin-1	165.2	7.6E-03	-1.2	-2.0	-1.7
P26772	Hspe1	10 kDa heat shock protein. mitochondrial	10.9	7.8E-03	1.1	2.5	2.2
P20850	Col9a1	Collagen alpha-1(IX) chain (Fragment)	31.2	7.9E-03	-1.3	-18.8	-14.2
Q07936	Anxa2	Annexin A2	38.7	8.1E-03	-1.1	-1.4	-1.4
O70351	Hsd17b10	3-hydroxyacyl-CoA dehydrogenase type-2	27.2	8.2E-03	1.0	1.5	1.5
Q07009	Capn2	Calpain-2 catalytic subunit	79.9	8.4E-03	-1.1	-1.5	-1.3
P07943	Akr1b1	Aldose reductase	35.8	8.5E-03	-1.1	-1.4	-1.3

HT, hydroxytyrosol; SEC, secoiridoids; MW, molecular weight; FC, fold change; N/A, not available.

The fold change is positive if the treatments are up-regulated and negative if they are down-regulated. comparing HT or SEC to control group and SEC to HT group.

Table 3. 34 significant proteins differentially expressed in heart tissue of healthy rats after HT or SEC treatments.

Swiss-Prot code	Gene symbol	Protein Name	MW (kDa)	*p*	FC (HT *versus* Control group)	FC (SEC versus Control group)	FC (SEC versus HT group)
Q9Z0W7	Clic4	Chloride intracellular channel protein 4	28.60	3.3E-05	-1.1	1.4	1.5
P15865	Hist1h1e	Histone H1.2	22.00	3.9E-05	-1.3	1.2	1.5
Q9QXQ0	Actn4	Alpha-actinin-4	104.80	4.0E-05	-1.1	1.3	1.5
O35952	Hagh	Hydroxyacylglutathione hydrolase. mitochondrial	34.10	1.6E-04	1.1	3.4	3.3
P0C0S7	H2afz	Histone H2A.Z	13.50	2.7E-04	-1.0	-1.4	-1.4
P43278	H1f0	Histone H1.0	20.90	3.1E-04	-1.2	1.3	1.5
Q5XIF6	Tuba4a	Tubulin alpha-4A chain	49.90	3.5E-04	1.1	1.2	1.1
P13084	Npm1	Nucleophosmin	32.50	5.0E-04	1.1	1.1	1.0
P15791	Camk2d	Calcium/calmodulin-dependent protein kinase type II subunit delta	60.00	5.6E-04	1.4	-1.4	-2.0
P62076	Timm13	Mitochondrial import inner membrane translocase subunit Tim13	10.50	6.6E-04	-1.2	-1.5	-1.3
Q9JLZ1	Glrx3	Glutaredoxin-3	37.80	6.8E-04	1.1	-1.3	-1.4
P00388	Por	NADPH--cytochrome P450 reductase	76.90	7.3E-04	-1.3	-1.4	-1.1
Q5XI73	Arhgdia	Rho GDP-dissociation inhibitor 1	23.40	7.7E-04	1.1	1.2	1.1
P83732	Rpl24	60S ribosomal protein L24	17.80	7.8E-04	-1.0	1.3	1.3
O70196	Prep	Prolyl endopeptidase	80.70	9.0E-04	1.0	-1.2	-1.3
P62919	Rpl8	60S ribosomal protein L8	28.00	1.0E-03	1.2	1.3	1.1
Q6RJR6	Rtn3	Reticulon-3	101.50	1.1E-03	1.0	1.2	1.2
P62329	Tmsb4x	Thymosin beta-4	5.00	1.2E-03	-1.2	1.9	2.3
P60901	Psma6	Proteasome subunit alpha type-6	27.40	1.3E-03	-1.2	1.1	1.4
P11030	Dbi	Acyl-CoA-binding protein	10.00	1.4E-03	-1.3	1.1	1.4
Q562C7	N/A	Pumilio domain-containing protein KIAA0020 homolog	72.70	1.6E-03	-1.2	1.2	1.5
P63159	Hmgb1	High mobility group protein B1	24.90	1.7E-03	-1.0	1.4	1.4
Q6AXV4	Samm50	Sorting and assembly machinery component 50 homolog	51.90	1.8E-03	1.1	-1.2	-1.3
P62898	Cycs	Cytochrome c. somatic	11.60	1.8E-03	-1.1	-1.3	-1.2
O35763	Msn	Moesin	67.70	1.9E-03	1.2	1.3	1.1
O88382	Magi2	Membrane-associated guanylate kinase. WW and PDZ domain-containing protein 2	141.00	2.1E-03	-1.0	-1.3	-1.3
Q62658	Fkbp1a	Peptidyl-prolyl cis-trans isomerase FKBP1A	11.90	2.2E-03	1.2	1.7	1.4
Q6P6R2	Dld	Dihydrolipoyl dehydrogenase. mitochondrial	54.00	2.2E-03	-1.1	-1.1	-1.0
Q6PCU8	Ndufv3	NADH dehydrogenase [ubiquinone] flavoprotein 3. mitochondrial	11.90	2.3E-03	-1.4	1.1	1.6
O35077	Gpd1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	37.40	2.5E-03	-1.2	-1.3	-1.1
O35303	Dnm1l	Dynamin-1-like protein	83.90	2.5E-03	-1.0	1.2	1.2
P08461	Dlat	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	67.10	2.5E-03	-1.1	-1.1	1.0
P02091	Hbb	Hemoglobin subunit beta-1	16.00	2.7E-03	-1.1	2.2	2.5
Q5M7W5	Map4	Microtubule-associated protein 4	110.20	2.7E-03	-1.2	1.0	1.2

HT, hydroxytyrosol; SEC, secoiridoids; MW, molecular weight; FC, fold change; N/A, not available.

The fold change is positive if the treatments are up-regulated and negative if they are down-regulated. comparing HT or SEC to control group and SEC to HT group.

Table 4. Functions and related proteins in aorta and heart tissue after HT or SEC treatments involved in the top-scored network: Cardiovascular System Development and Cancer

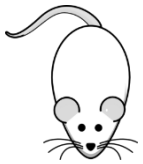
	Cardiovascular System Functions	Aorta tissue proteins	Heart tissue proteins
Cardiac functions	Formation of Thrombus	Anxa2	-
	Occlusion of blood vessel	Kng1/Kng1l1	-
	Cell viability of cardiomyocytes	Hk2, Hspd1, Hk2	-
	Survival of ventricular myocytes	Hk2,	-
	Proliferation of endothelial cells	Gja1, Kng1/Kng1l1	-
	Cardiogenesis	Gja1, Mylk3	-
	Vasoconstriction of blood vessel	Gja1, Hspa1a/Hspa1b	-
	Migration of endothelial cell lines	Rac1	-
	Heart rate	Rac1, Tpm1	-
	Cell death of cardiomyocytes	Hspd1, Hspe1	-
	Apoptosis of cardiomyocytes	Hspd1, Hspe1	-
	Hypertrophy of cardiomyocytes	Cryab	Camk2d, H2afz, Glrx3
	Pericarditis	-	Tuba4a
	Myocardial infarction	-	Tuba4a, Psma6
	Acute myocardial infarction	-	Tuba4a
	Hypertrophy of heart	-	Camk2d, Fkbp1a, H2afz, Glrx3
	Severe heart failure	-	Camk2d
	Failure of heart	-	Camk2d, Fkbp1a
	Hypertrophy of left ventricle	-	Fkbp1a
	Congestive heart failure	-	Fkbp1a
	Systolic dysfunction	-	Fkbp1a
Lipid metabolism	Synthesis of acetyl-coenzime A	-	Dlat, Dld,
	Fatty acid metabolism	-	Dlat, Dld, Dbi, Hbb, Msn, Por
	Acetylation of acetyl-coenzyme A	-	Dlat
	Acetylation of dihydrolipoic acid	-	Dlat
	Synthesis of lipid	-	Dlat, Dld, Dbi, Hbb, Por
	Synthesis of fatty acid	-	Dlat, Dld, Hbb
	Concentration of cholesterol	-	Dbi, Por, Arhgdia
	Quantity fo very long chain fatty acid	-	Dbi
	Quantity of farnesyl pyrophosphate	-	Por
	Oxidation of testosterone	-	Por
	Conversion of androstenedione	-	Por
	Concentration of tretinoin	-	Por
	Concentration of retinol	-	Por
	Cytotoxicity of tacrolimus	-	Fkbp1a
	Oxidation of cardiolipin	-	Cycs
	Oxidation of linoleic acid	-	Cycs
	Binding of paclitaxel	-	Map4

Experimental animal design

Tissue and protein extraction

Protein digestion

TMT-10 plex labelling



♀ Wistar rats n=12

Control group (n=4)

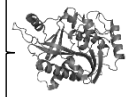
HT group (n=4)

SEC group (n=4)

aorta

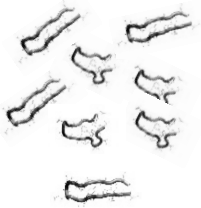
heart

Collagenase incubation (aorta)
SDS-Lysis

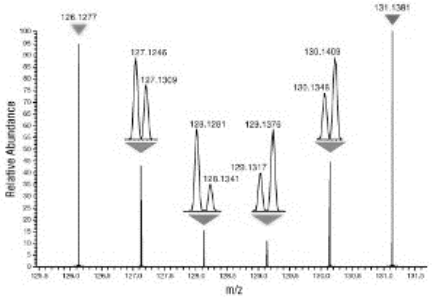


proteins

LysC/Tryp digestion on FASP



peptides



Labelled peptides

Peptide IEF fractionation

RP fractionation & MS/MS analysis

Peptide/Protein ID & Quantification

Pathways analysis

Diseases and functions

Biomarker applications

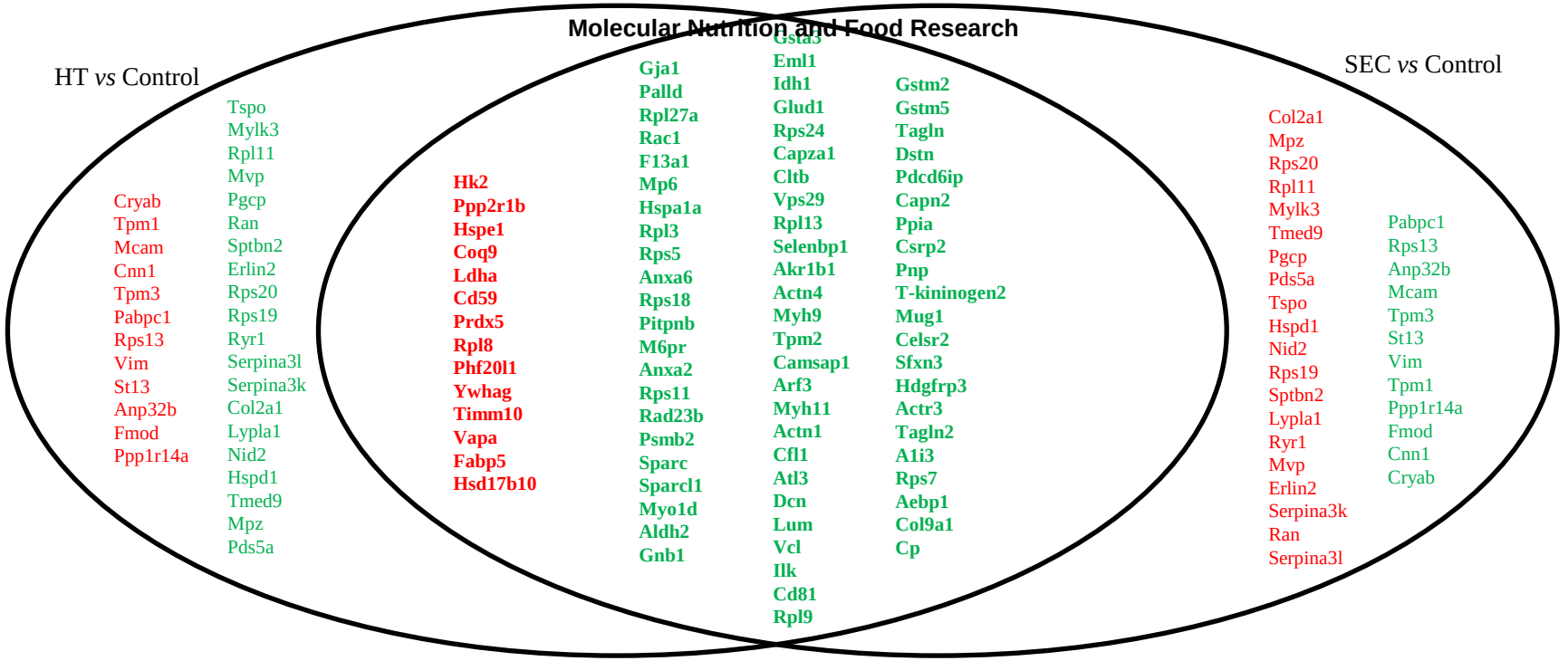
IPA® INGENUITY PATHWAY ANALYSIS

Proteome Discover 1.4
Mascot 2.5

nanoLC-(Orbitrap) MS

Off-Gel

A


HT *vs* Control
SEC *vs* Control
Cryab
Tpm1
Mcam
Cnn1
Tpm3
Pabpc1
Rps13
Vim
St13
Anp32b
Fmod
Ppp1r14a
Tspo
Mylk3
Rpl11
Mvp
Pgcp
Ran
Sptbn2
Erlin2
Rps20
Rps19
Ryr1
Serpina3l
Serpina3k
Col2a1
Lypla1
Nid2
Hspd1
Tmed9
Mpz
Pds5a
Hk2
Ppp2r1b
Hspe1
Coq9
Ldha
Cd59
Prdx5
Rpl8
Phf20l1
Ywhag
Timm10
Vapa
Fabp5
Hsd17b10
Gja1
Palld
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Rac1
F13a1
Mp6
Hspa1a
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Rps18
Pitpnb
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Anxa2
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Rad23b
Psmb2
Sparc
Sparcl1
Myo1d
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Gnb1
Gsta3
Eml1
Idh1
Glud1
Rps24
Capza1
Cltb
Vps29
Rpl13
Selenbp1
Akr1b1
Actn4
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Tpm2
Camsap1
Arf3
Myh11
Actn1
Cfl1
Atl3
Dcn
Lum
Vcl
Ilk
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Dstn
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Sptbn2
Lypla1
Ryr1
Mvp
Erlin2
Serpina3k
Ran
Serpina3l
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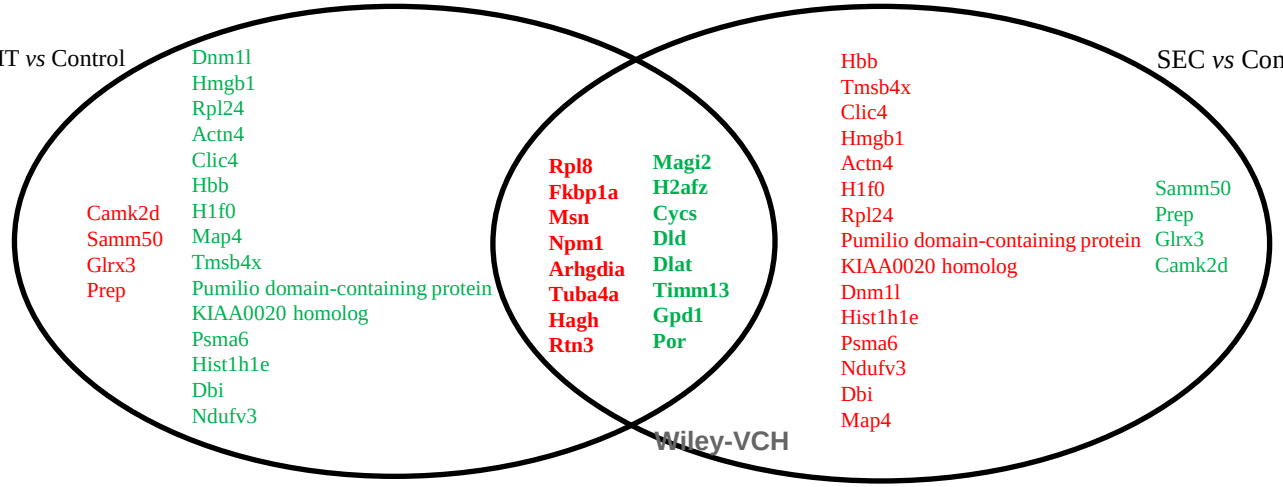
Fmod
Cnn1
Cryab

B


HT *vs* Control
SEC *vs* Control
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Glrx3
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Rpl24
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Hbb
H1f0
Map4
Tmsb4x
Pumilio domain-containing protein
KIAA0020 homolog
Psma6
Hist1h1e
Dbi
Ndufv3
Rpl8
Fkbp1a
Msn
Npm1
Arhgdia
Tuba4a
Hagh
Rtn3
Magi2
H2afz
Cycs
Dld
Dlat
Timm13
Gpd1
Por
Hbb
Tmsb4x
Clic4
Hmgb1
Actn4
H1f0
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Pumilio domain-containing protein
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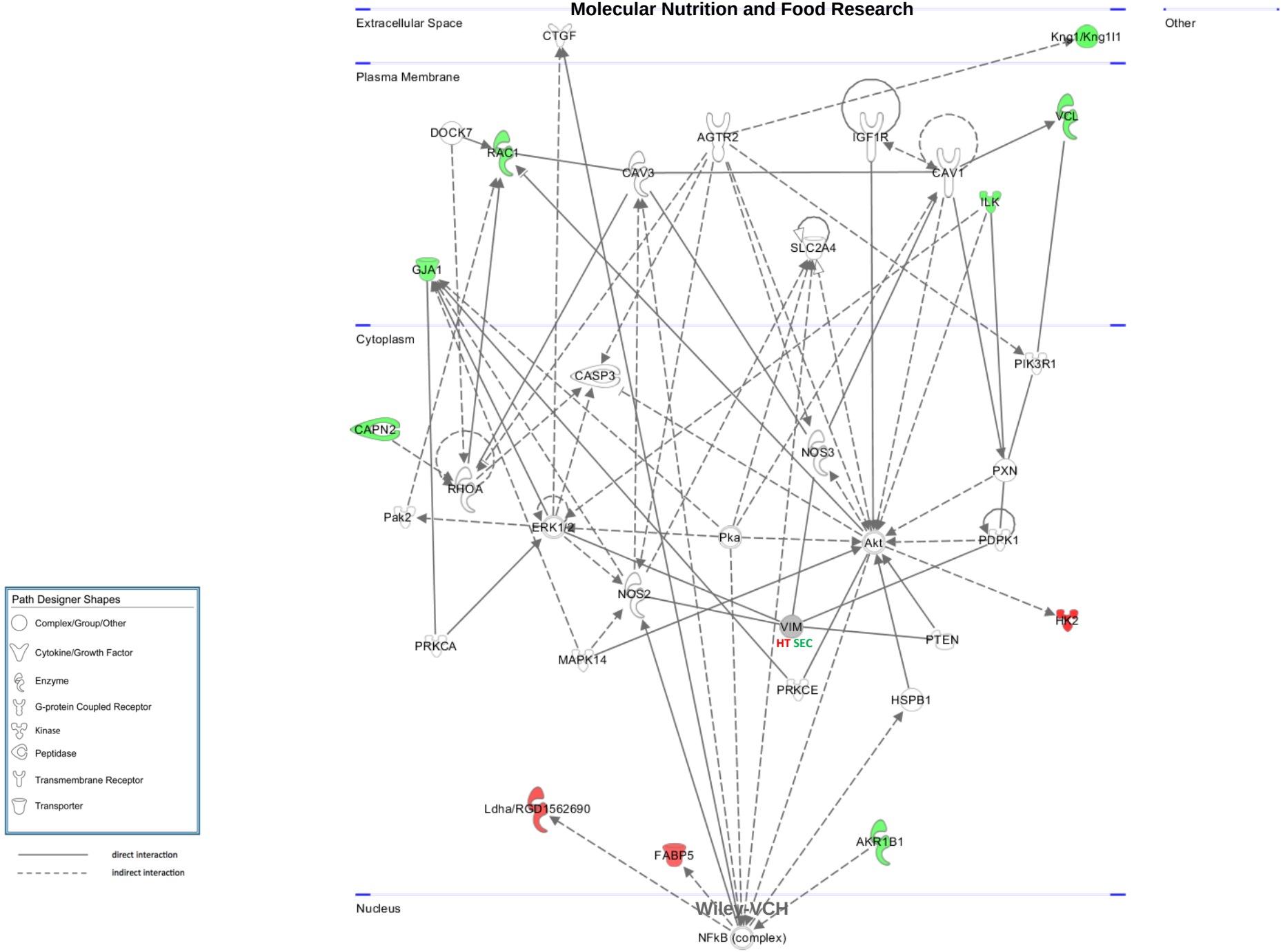
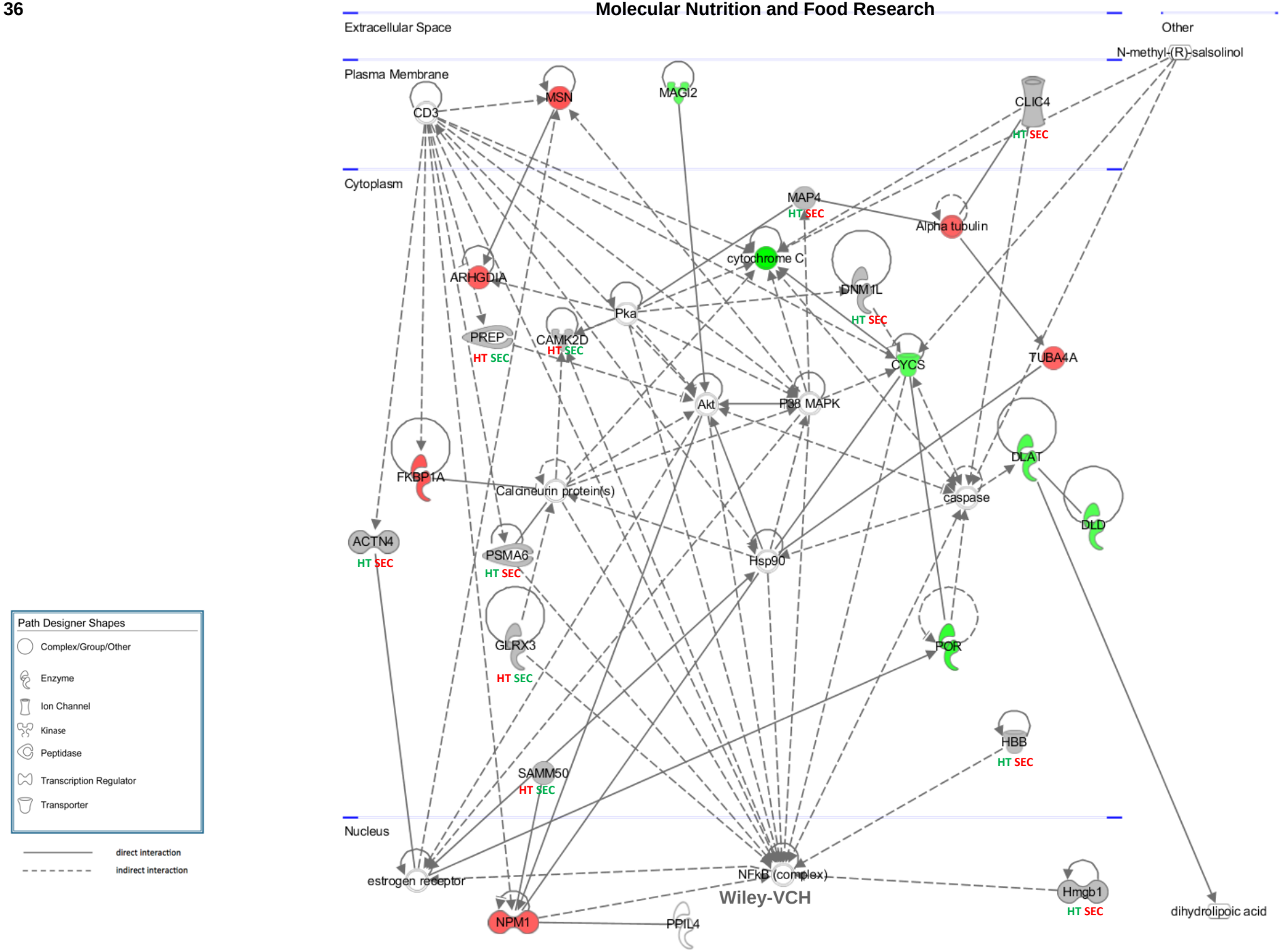
Camk2d


Extracellular Space
Other
Plasma Membrane
Cytoplasm
Nucleus
CTGF
Kng1/Kng1l1
DOCK7
RAC1
AGTR2
IGF1R
VCL
CAV3
CAV1
ILK
SLC2A4
GJA1
PIK3R1
CASP3
CAPN2
NOS3
PXN
RHOA
Pak2
ERK1/2
Pka
Akt
PDPK1
NOS2
HK2
VIM
HT SEC
PTEN
PRKCA
MAPK14
PRKCE
HSPB1
Ldha/RGD1562690
AKR1B1
FABP5
NFkB (complex)
Path Designer Shapes
Complex/Group/Other
Cytokine/Growth Factor
Enzyme
G-protein Coupled Receptor
Kinase
Peptidase
Transmembrane Receptor
Transporter
direct interaction
indirect interaction
Wiley-VCH


Extracellular Space
Other
N-methyl-(R)-salsolinol
Plasma Membrane
MSN
MAGI2
CD3
CLIC4
HT SEC
Cytoplasm
MAP4
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cytochrome C
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DNM1L
HT SEC
Pka
PREP
HT SEC
CAMK2D
HT SEC
CYCS
TUBA4A
Akt
P38 MAPK
DLAT
FKBP1A
Calcineurin protein(s)
caspase
DLD
ACTN4
HT SEC
PSMA6
HT SEC
Hsp90
GLRX3
HT SEC
POR
HBB
HT SEC
SAMM50
HT SEC
Nucleus
estrogen receptor
NFkB (complex)
Hmgb1
HT SEC
dihydrolipoic acid
NPM1
PPIL4
Wiley-VCH
Path Designer Shapes
Complex/Group/Other
Enzyme
Ion Channel
Kinase
Peptidase
Transcription Regulator
Transporter
direct interaction
indirect interaction