## Original Article Ghrelin-mediated pathway in Apolipoprotein-E deficient mice: a survival system

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Abstract: Renal diseases interfere with the regulation of several metabolic pathways including dyslipidemia. The latter includes increased triglycerides, very low-density lipoprotein levels and decreased high-density lipoproteins. These lipoproteins change during renal injury. Apolipoprotein-E deficient mice (ApoE<sup>-/-</sup>) are considered a very well accepted model of hypercholesterolemia with marked renal pathological alterations. Ghrelin hormone is mainly secreted from the stomach when the stomach is empty, but it is also found in the kidney. In this organ it has autocrine and/or paracrine roles determining glomerular filtration rate, tubular phosphate and sodium reabsorption. Interestingly, it has been demonstrated that ghrelin levels increase after fasting. This mechanism induces an interaction with sirtuin 1 (SIRT1)/p53 pathway suggesting a link between ghrelin and SIRT1 in the regulation of salt and water metabolism. The mechanisms of ghrelin-induced SIRT1 expression are not yet fully understood. Recent studies indicate that SIRT1 exerts renoprotective properties against kidney diseases. This could be a very interesting point for underlining the important role of the ghrelin-SIRT1 system. Water movement across biological cell membranes is enhanced or facilitated by tetrameric membrane-bound channels, named aquaporin (AQP) family, and in particular, AQP1 and AQP2 proteins. In this study, we evaluated the possible pathway existing among the ghrelin/SIRT1/AQP1/AQP2 system in APOE<sup>-/-</sup> mice in order to clarify or stress the role played by said system in renal diseases associated to aging with or without comorbities. The results could provide a basis for considering ghrelin as a new target for therapeutic strategies of renal injury.

Keywords: Renal diseases, ApoE<sup>-/-</sup> mice, ghrelin, SIRT1, AQP1, AQP2

#### Introduction

Renal diseases and, in particular, chronic kidney diseases (CKD) affect millions of patients and increase the risk of cardiovascular diseases and death [1, 2]. It is known that renal diseases interfere with the regulation of several metabolic pathways. They also play an important role in the development of dyslipidemia, deregulating the levels or metabolism of many molecules. The relationship between dyslipidemia and renal diseases is reciprocal since dyslipidemia is a risk factor for renal pathology, and this latter causes several alterations on lipoprotein profile [3]. This profile includes increased triglycerides, very low-density lipoprotein levels and decreased high-density lipoproteins. The lipoproteins change during renal injury, and therefore, a better understanding of the drivers and consequences of these changes will help to identify new biomarkers for optimizing therapeutic approaches to these diseases [2].

Apolipoprotein-E deficient (ApoE<sup>-/-</sup>) mice are considered a very well accepted model of hypercholesterolemia [4]. In these mice, dyslipidemia is associated to marked renal pathological alterations, including mesangial expansion, an increase of extracellular matrix area [5], and lipid deposition in the glomerular capillaries. The latter feature is typically found in glomerulopathy, a renal disease associated to specific ApoE mutations in humans [6]. It should be noticed that many evidences suggested that lipid accumulation in the kidney contributes to the progression of CKD [7].

Ghrelin hormone, a 28 amino acid peptide hormone, is mainly secreted from the stomach when the stomach is empty [8], but it is also found in the kidney, where its levels are much higher than in plasma. These findings suggest that renal ghrelin has autocrine and/or paracrine roles [9], and is able to increase renal blood flow, glomerular filtration rate, tubular phosphate and sodium reabsorption [9]. In particular, ghrelin stimulates distal nephrondependent sodium reabsorption by enhancing the trafficking of epithelial sodium channels (ENaCs) to the apical membrane in vivo [10-12]. Moreover, it reduces water intake under certain circumstances, especially when drinking is induced by a single acute stimulus [13]. Recently, it has been demonstrated that ghrelin attenuates ureteral obstruction-induced fibrosis by inhibiting NLR family pyrin domain-containing protein 3 inflammosome and endoplasmic reticulum stress in vivo [14]. Interestingly, it has been demonstrated that ghrelin levels increase after fasting and this induces an interaction with sirtuin 1 (SIRT1)/p53 pathway [15]. This data suggests a link between ghrelin and SIRT1 in the regulation of salt and water metabolism [16]. Of note, the mechanisms by which ghrelin-induced SIRT1 expression are not yet fully understood. Recent studies have indicated that SIRT1 exerts renoprotective properties against kidney diseases [17], and this could be a very interesting point for underlining the important role of the ghrelin-SIRT1 system. These proteins are important for movement of water not only in a single cell of some tissues but also in many organs and in the full body. Water movement across biological cell membranes is enhanced or facilitated by tetrameric membrane-bound channels, named aquaporin (AQP) family [18]. In particular, AQP1 has been identified in cortical and medullary structures whereas AQP2 has been found only in Henle loops and collecting ducts in renal medulla [19].

On the basis of these considerations, in this study we evaluated the possible pathway existing among the Ghrelin/SIRT1/AQP1/AQP2 system in ApoE<sup>-/-</sup> mice in order to clarify or stress the role played by said system in renal diseases. The results could provide a basis for considering ghrelin as a new target for therapeutic strategies in renal injury.

#### Materials and methods

#### Experimental groups

Eighteen C57BL/6 control mice and eighteen ApoE<sup>-/-</sup> mice were housed in an animal experimental unit with 12 hours alternating light-dark cycle and constant temperature. All the experimental animals were fed with standard chow diet and water ad libitum. Mice were bred in the Animal Care Facility of the Department of Human Nutritional Sciences of the University of Manitoba (Winnipeg, MB, Canada) and the committee for Supervision of Animal Experiments of the University of Manitoba (Winnipeg, MB, Canada) approved the protocols of this study. Mice were randomly divided into six groups (six animals each): C57BL/6 controls sacrified at 6 weeks of age; C57BL/6 controls sacrified at 15 weeks of age; C57BL/6 controls sacrified at 15 months (60 weeks) of age; ApoE<sup>-/-</sup> sacrified at 6 weeks of age; ApoE<sup>-/-</sup> sacrified at 15 weeks of age and ApoE<sup>-/-</sup> sacrified at 15 months (60 weeks) of age. At the end of each different experimental period, mice were sacrified under local ether anaesthesia using a nosal cone. Kidneys were rapidly dissected, fixed in 10% neutral buffered formalin for 24 hours, dehydrated in serial alcohol solutions and embedded in paraffin wax, according to standard procedures [20, 21]. Serial sections were used for the morphological and immunohistochemical evaluations.

#### Renal histological assessment

Serial kidney sections (7 µm thick) of each sample were cut with a microtome and placed on glass slides. Alternate sections were deparaffinised in xylene and hydrated in serial alcohol solutions. Thus, they were stained with haematoxylin eosin (Bio Optica, Milan, Italy), and observed by an optical light microscope (Olympus, Hamburg, Germany) at the final magnification of 200×.

## Immunolocalization of ghrelin, SIRT1, AQP1 and AQP2

Sections from kidney of ApoE<sup>-/-</sup> and C57BL/6 mice were cut (7 µm thick) and studied using immunohistochemical methods in order to evaluate Ghrelin, SIRT1, AQP1 and 2 expression in both cortex and medulla. Serial sections were deparaffinized in xylene, using serial alco-

hol solutions and rinsed with distilled water. Sections were subjected to antigen retrieval in 0.01 M sodium citrate buffer, pH 6.0, in a microwave oven: one cycle of 3 min at 600 Watts [22]. They were later washed in Tris-buffered saline (TBS) for 5 minutes, incubated in 3% hydrogen peroxide, blocked with 1% bovine serum albumin for 1 hour at room temperature and finally incubated for three hours at room temperature with the following primary antibodies: rabbit monoclonal anti-ghrelin (diluted 1:500, Abcam, Cambridge, UK), rabbit polyclonal anti-SIRT1 (diluted 1:200; Santa Cruz Biotechnology Inc., Dallas, TX, USA), rabbit polyclonal anti-AQP1 (diluted 1:400; Chemicon International Inc., Temecula, CA, USA) and rabbit polyclonal anti-AQP2 (diluted 1:500; Chemicon International Inc., Temecula, CA, USA). Then, the samples were washed in TBS (5 minutes) and labelled with secondary antibody, an anti-rabbit biotinylated immunoglobulin (Dako, Glostrup, Denmark), and successively conjugated with avidin-biotin peroxidase complex. The reaction products were visualized using 0.33% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), as chromogen. The sections were finally counterstained with haematoxylin (Bio Optica, Milan, Italy), dehydrated and mounted [23, 24]. The immunohistochemical controls were performed by omitting the primary antibody and in isotype matched IgG presence.

## Semi-quantitative and quantitative analyses

The immunohistochemical data for renal ghrelin, SIRT1, AOP1 and AOP2 were evaluated by a blinder examiner and were expressed as negative (-), very weak (-/+), weak (+), moderate (++), and strong (+++) positivity. Additionally, we evaluated immunostaining as percentage of area (%), for the same proteins. In particular, staining intensity was measured 20 random fields in six samples for each experimental group with the same area (0.04  $\mu$ m<sup>2</sup>/field) [25, 26]. They have been used for calculating the percentage of arbitrary areas. Digitally fixed images of the slices at 200× magnification were analysed using an optical microscope (Olympus, Hamburg, Germany) equipped with an image analyser (Image Pro Premier 9.1, MediaCybernetics, Rockville, MD, USA). The data were pooled to represent a mean value and a statistical analysis was applied to compare the results obtained from the different experimental groups.

### Western blot ghrelin evaluation

The kidney homogenates of each experimental groups were loaded into 10% SDS-polyacrylamide gels and subjected to electrophoresis. The separated proteins were transferred to nitrocellulose membranes and then incubated with bovine albumin serum solution for 1 hour, followed by overnight incubation at 4°C with mouse monoclonal anti-β-actin antibody (diluted 1:5000; AC5441-Sigma Aldrich, St. Louis, MO, USA) and rabbit monoclonal ghrelin antibody (diluted 1:450; Abcam, Cambridge, UK). Then, protein detection was carried out using secondary infrared fluorescent dye conjugated antibodies absorbing at 800 nm or 700 nm. Finally, the blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Inc., Lincoln, USA).

#### Statistical analyses

Results are expressed as mean ± S.E.M. Statistical significance of differences among the experimental groups for all the markers was evaluated by analysis of variance (one way ANOVA calculated by Origin ® 7SRI, 1991-2002 OriginLab Corporation, One Roundhouse Plaza, Northampton, MA 01060 USA) corrected by Bonferroni test with significance set at P<0.05. The results of semi-quantitative immunohistochemical evaluation were compared between both young and old controls and ApoE<sup>-/-</sup> mice at the same age.

## Results

Morphological evaluations were similar, both in cortex and medulla, in young control mice (6 weeks and 15 weeks old) and, also, in young ApoE<sup>-/-</sup> animals (6 weeks and 15 weeks old). Immunohistochemical findings for ghrelin-SIRT1-AQP1 and AQP2 proteins showed the same results observed by morphological evaluations. It is important to underline that the positivity decreased quickly in young and old animals from all experimental groups. Thus, we decided to show and describe only morphological and immunohistochemical evaluations of young animals (6 weeks old) compared to old control and ApoE<sup>-/-</sup> mice (15 months-60 weeksold) because we considered this age more significant for explaining our results.

## HAEMATOXYLIN EOSIN



**Figure 1.** Haematoxylin-eosin staining showed the morphological relationship among control 6 weeks (A, C) and 15 months (60 weeks) old (B, D) mice, ApoE<sup>-/-</sup> 6 weeks (E, G) and 15 months (60 weeks) old (F, H) mice (200X). The morphological evaluations showed glomeruli (G), proximal (PT), and distal (DT) tubules, Henle loops (HL) and collecting ducts (CD) respectively in renal cortex and medulla. The cytoarchitecture changed with aging in control mice (15 months old) and in ApoE<sup>-/-</sup> mice (6 and 15 weeks old). Necrosis, swelling of tubular epithelial cells (black rectangles and head arrows) and epithelial cell deposits (arrows) have been found both in cortex and medulla in old control animals and in young and old ApoE<sup>-/-</sup> mice.

#### Renal histopathological evaluation

Haematoxylin and eosin staining from young control group showed a normal morphology both in cortex and medulla. In particular, cortex from young controls showed normal glomeruli and tubular structures. Moreover, the latter had very few epithelial cell necrosis as shown in **Figure 1A**. Instead, in old control animals (15 months old) the cytoarchitecture was changed

and damaged. Cortex showed necrosis and swelling of epithelial cells in proximal and distal tubules and endothelial/mesangial cell necrosis in glomeruli (Figure 1B). About medulla, young controls showed normal collecting ducts and Henle loops (Figure 1C). Old animals had necrosis and swelling in these structures and several deposits inside their lumen (Figure 1D). On the contrary young and old ApoE<sup>-/-</sup> mice showed interstitial inflammation, tubular epithelial cell death and glomerulosclerosis that were more evident in old animals. In detail, the kidney of animals (6 weeks old) had cortical changes consisting in tubular and glomerular injury with dilatation of the lumen and Bowman's capsule respectively (Figure 1E). The dilatation of these structures increases with age of animals as shown in Figure 1F. Moreover, proximal and distal tubules showed reduction in the number and swelling of the epithelial cells (Figure 1E, 1F). These alterations are similar to that observed in medullary collecting ducts and Henle loops. These injures were more severe in old mice compared to young animals (Figure 1G, 1H). Remarkable, cortical and medullary damages were more evident in kidney of old ApoE<sup>-/-</sup> mice compared to old control groups.

## Immunolocalization of ghrelin in kidney

Immunohistochemical analysis demonstrated that ghrelin was expressed in renal cortex and medulla of young control and ApoE<sup>-/-</sup> animals. Instead, old ApoE<sup>-/-</sup> mice did not have ghrelin positivity respect to that observed in control groups at the same age.

#### Renal cortex

Ghrelin expression was clearly observed in cytoplasm of tubular epithelial cells and in endothelial cells of glomeruli. Furthermore, it is important to underline that the observed positivity was more evident in tubular structures than in glomeruli. This positivity was strong in distal tubules, moderate in proximal tubules and weak in glomeruli of young control mice (Figure 2A). Instead, ghrelin expression was weak and sometimes moderate in all tubular structures and always weak/very weak in glomeruli of old control mice (Figure 2B). Ghrelin positivity was already lower in young ApoE<sup>-/-</sup> animals compared to control animals at the same age. After this time, the expression decreased very quickly and it was no evident in ApoE<sup>-/-</sup> old animals. In detail, glomeruli and tubular structures of young ApoE<sup>-/-</sup> mice had respectively weak and weak/moderate positivity, whereas ghrelin expression was negative in old animals. The semi-quantitative observations reported above are summarized in **Table 1**.

#### Renal medulla

Ghrelin expression was moderately and weakly evident in cytoplasm of medullary structures in young control and ApoE<sup>-/-</sup> animals. Very weak ghrelin positivity was observed in old controls, whereas no positivity was observed in old ApoE<sup>-/-</sup> mice. Remarkable, ghrelin expression decreased quickly both in control and ApoE<sup>-/-</sup> mice from 6 to 15 weeks and this decrease was still more evident after this age. The quantitative results were reported as graph in **Figure 3A**.

#### Immunolocalization of SIRT1 in kidney

Immunohistochemical evaluation showed that SIRT1 was differently expressed in renal cortex and medulla of control and ApoE<sup>-/-</sup> mice. Moreover, the changes of SIRT1 expression were similar but with different positivity among control and ApoE<sup>-/-</sup> mice both in young and old animals.

#### Renal cortex

In young control mice, SIRT1 was weakly and sometimes moderately expressed in glomeruli but strongly positive in cytoplasm of proximal and distal tubules (**Figure 2E**). Instead, it was weakly and sometimes moderately expressed in glomeruli and tubular structures of old control group (**Figure 2F**).

In young ApoE<sup>-/-</sup> mice SIRT1 was weakly expressed in glomeruli and moderately positive in cytoplasm of tubular structures, whereas it was completely negative in old ApoE<sup>-/-</sup> animals (**Figure 2G, 2H**). Interestingly, cytoplasmic positivity was lower in young ApoE<sup>-/-</sup> mice compared to control mice at the same age.

Of note, SIRT1 expression was evident in young control and ApoE<sup> $\gamma$ </sup> mice even if they showed a different intensity of positivity.

The semi-quantitative observations reported above are summarized in **Table 2**.



**Figure 2.** Immunohistochemistry of ghrelin, SIRT1 and AQP1 showed a different positivity in the renal cortex of control (6 weeks and 15 weeks-60 weeks-old) (A, B, E, F, I, J) mice, in ApoE<sup>/-</sup> 6 weeks and 15 months (60 weeks) old (C, D, G, H, K, L) mice (200X). The positivity of these proteins was seen in glomeruli (G), distal (DT) and proximal (PT) tubules both in young and old mice; instead the expression of these proteins was evident in young and not in old ApoE<sup>/-</sup> mice.

**Table 1.** Semi-quantitative analysis of ghrelin expression in renal cortex of both control and ApoE<sup>-/-</sup> mice (6 weeks and 15 months-60 weeks-old)

|                  | ,                  |                      |                                    |                                      |
|------------------|--------------------|----------------------|------------------------------------|--------------------------------------|
|                  | CTR 6<br>weeks old | CTR 15<br>months old | ApoE <sup>-/-</sup> 6<br>weeks old | ApoE <sup>-/-</sup> 15<br>months old |
| Glomeruli        | +                  | +\+/-                | +                                  | -                                    |
| Proximal tubules | ++                 | +/++                 | +/++                               | -                                    |
| Distal tubules   | +++                | +/++                 | +/++                               | -                                    |
|                  |                    |                      |                                    |                                      |

Note. The data are expressed as negative (-), very weak (+/-), weak (+), moderate (++), strong (+++) positivity; CTR: C57BL/6 mice.

#### Renal medulla

Immunohistochemical analysis revealed strong and weak cytoplasmic expression of SIRT1 in Henle loops and collecting ducts of young and old controls respectively. As observed for cortical positivity of SIRT1, aging decreases significantly its expression also in medulla of ApoE<sup>-/-</sup> groups. Its positivity was



Figure 3. Renal quantitative analyses in control 6 weeks and 15 months (60 weeks) old mice, in ApoE<sup>-/-</sup> 6 weeks and 15 months (60 weeks) old mice respectively for ghrelin 1 (A), SIRT1 (B) and AQP1 (C). The results showed a significant decrease of protein positivity with aging both in control and ApoE<sup>-/-</sup> mice. + P<0.05 vs C57BL/6, 6 weeks old; \*P<0.05 vs C57BL/6, 15 weeks old; #P<0.05 vs C57BL/6, 15 months (60 weeks) old.

already reduced respect to that observed in control mice at the same age. In fact, SIRT1 expression declines gradually increasing the age of experimental mice, reaching no positivity in old ApoE<sup>-/-</sup> animals. The quantitative observations are summarized in **Figure 3B** and they are similar to that observed for ghrelin quantitative data reported in **Figure 3A**.

# Immunolocalization of aquaporin1 and 2 in kidney

AQP1 was expressed both in cortex and medulla, whereas AQP2 was localized only in the medulla.

#### Renal cortex

In young control mice AQP1 was moderately expressed in cytoplasm of proximal tubules, very weak evident in endothelial cells of glomeruli and no present in distal tubules (Figure 21). Its positivity was weak/very weak in old control animals (Figure 2J). Young ApoE<sup>-/-</sup> showed weak/very weak cytoplasmic positivity in all renal structures (Figure 2K); instead no positivity was observed in old ApoE-/- mice (Figure 2L). Interestingly, in these animals, the expression of AQP1 was lower also respect to young and old control groups. The semi-quantitative observations reported above are summarized in Table 3.

## Renal medulla

AQP1 and AQP2 expression was localized in cytoplasmic of collecting ducts and Henle loops and the proteins had same positivity. Thus, quantitative analysis of AQP1 positivity was visualized in **Figure 3C**; semi-quantitative and quantitative analyses of AQP2 expression were reported in **Table 4** and **Figure 4**. The quantitative analysis for AQP1 showed a decreased positivity

in medullary structures of control and ApoE<sup>-/-</sup> mice due to aging even if the latter animals had a lower positivity already at young age (**Figure 3C**). AQP2 positivity was respectively strongly/ moderately and weakly positive in medulla of young and old controls (**Figure 4A**, **4B**). Instead its positivity was moderate in young ApoE<sup>-/-</sup> mice and not present in old ApoE<sup>-/-</sup> groups

| Table 2. Semi-quantitative analysis of SIRT1 expression in             |
|------------------------------------------------------------------------|
| renal cortex of both control and ApoE <sup>-/-</sup> mice (6 weeks and |
| 15 months-60 weeks-old)                                                |

|                  | CTR 6     | CTR 15     | ApoE <sup>-/-</sup> 6 | ApoE <sup>-/-</sup> 15 |
|------------------|-----------|------------|-----------------------|------------------------|
|                  | weeks old | months old | weeks old             | months old             |
| Glomeruli        | +/++      | +/++       | +                     | -                      |
| Proximal tubules | +++       | +/++       | ++                    | -                      |
| Distal tubules   | +++       | +/++       | ++                    | -                      |

Note. The data are expressed as negative (-), very weak (+/-), weak (+), moderate (++), strong (+++) positivity; CTR: C57BL/6 mice.

**Table 3.** Semi-quantitative analysis of AQP1 expression in renal cortex of both control and ApoE<sup>-/-</sup> mice (6 weeks and 15 months-60 weeks-old)

|                  | ,         |            |                       |                        |
|------------------|-----------|------------|-----------------------|------------------------|
|                  | CTR 6     | CTR 15     | ApoE <sup>-/-</sup> 6 | ApoE <sup>-/-</sup> 15 |
|                  | weeks old | months old | weeks old             | months old             |
| Glomeruli        | +/-       | +\+/-      | +\+/-                 | -                      |
| Proximal tubules | ++        | +\+/-      | +\+/-                 | -                      |
| Distal tubules   | -         | +\+/-      | +\+/-                 | -                      |

Note. The data are expressed as negative (-), very weak (+/-), weak (+), moderate (++), strong (+++) positivity; CTR: C57BL/6 mice.

**Table 4.** Semi-quantitative analysis of AQP2 expression in renal medulla of both control and ApoE<sup>-/-</sup> mice (6 weeks and 15 months-60 weeks-old)

|                  |           | ,          |                       |                        |
|------------------|-----------|------------|-----------------------|------------------------|
|                  | CTR 6     | CTR 15     | ApoE <sup>-/-</sup> 6 | ApoE <sup>-/-</sup> 15 |
|                  | weeks old | months old | weeks old             | months old             |
| Henle loops      | +++/++    | +          | ++                    | -                      |
| Collecting ducts | +++/++    | +          | ++                    | -                      |

Note. The data are expressed as negative (-), very weak (+/-), weak (+), moderate (++), strong (+++) positivity; CTR: C57BL/6 mice.

(**Figure 4C**, **4D**). The quantitative observations are summarized in **Figure 4E**.

Noteworthy, AQP1 and 2 patterns of positivity were similar to that observed for ghrelin-SIRT1 system decreasing with aging in all experimental groups. Furthermore, young ApoE<sup>-/-</sup> mice showed a lower degree of positivity respect to control animals at the same age.

#### Western blot ghrelin evaluation

As previously observed by immunohistochemistry analyses, the western blot analyses **con**firmed that ghrelin was strongly expressed at kidney level of young control mice, whereas its expression decreased significantly in old control mice. Notably, renal ghrelin expression of young ApoE<sup>-/-</sup> mice was lower respect to agematched control young mice and further decreased, becoming almost undetectable, in old ApoE<sup>-/-</sup> mice. These observations are summarized in Figure 5.

#### Discussion

This study provides evidence that: 1-ghrelin is differently expressed in cortex and medulla of young and old animals as well as between control and ApoE<sup>-/-</sup> mice; 2-SIRT1, AQP1 and AQP2 have a similar positivity pattern in control and ApoE<sup>-/-</sup> mice at different ages, but their expression decreases with aging in all experimental groups, similarly to ghrelin.

The ghrelin immunopositivity results support the findings indicating that it is locally produced in the kidney and may have a direct effect on this organ [9, 27]. Furthermore, Takeda et al. [28] demonstrated, in an experimental model of ischemic acute renal failure, that ghrelin administered both before and during ischemia decreased renal tissue injury and improved excretory function. These effects were associated with endotheliumdependent vasodilation, with nitric oxide increase thus leading to a decrease in oxidative stress, which is thought to have a central role in ischemia-reperfusion injury [29]. After

this renal injury, tubular epithelial cell apoptosis has been reported [27, 30, 31], but ghrelin has been shown to decrease the apoptosis of these cells. This could be due to increase of insulin-like growth factor 1 (IGF-1) that has antiapoptotic properties in several ischemia-reperfusion injury models [28, 30, 32]. Moreover, Ariyasu et al. [33] showed that the mean plasma concentrations of ghrelin in normal-weight elderly people were lower than those in younger people. These ApoE<sup>-/-</sup> mice of the same age have less ghrelin positivity compared to young animals (6, 15 weeks old). These findings have been also confirmed by western blot assay. It is of note that aging is often associated with a progressive dysfunction in energy metabolism. an increased homeostatic imbalance and a risk of diseases [34-37]. The reasons for aging-related diseases are multifactorial; among these



AQP2

Figure 4. AQP2 immunohistochemistry of control 6 weeks and 15 months (60 weeks) old (A, B) mice, of ApoE<sup>/-</sup> 6 weeks and 15 months (60 weeks) old (C, D) mice (200X). The positivity was seen both in Henle loops (HL) and collecting ducts (CD). Quantification of AQP2 positivity in the groups reported above (E) showed a significant decrease with the aging. + P<0.05 vs C57BL/6, 6 weeks old; \*P<0.05 vs C57BL/6, 15 weeks old; #P<0.05 vs C57BL/6, 15 months (60 weeks) old.

factors, the decrease of ghrelin expression, as shown in this study, could play an important role. These findings agree with those obtained by Wolden-Hanson [38], showing that fasting failed to induce increased ghrelin in aged rats compared to young animals. Moreover, it has been demonstrated that ghrelin administration restored a young adult phenotype in liver of old mice [39, 40]. On the basis of these considerations, it seems that ghrelin decreases with aging and as a result of several diseases. This could contribute to the risk of various human



Figure 5. Representative western blot assay shows renal ghrelin expression of control (6 weeks and 15 months-60 weeks-old) mice and of ApoE<sup> $\gamma$ </sup> (6 weeks, 15 months-60 weeks-old) animals.

pathologies, similarly to that observed by Viser et al. [41] in elderly women. In fact, these Authors suggested that ghrelin mimetics extend health span by increasing IGF-1 and lowering IL-6.

Interestingly, the data presented in this paper also demonstrate that ghrelin is evident not only in tubular structures but also in glomeruli of young control animals and ApoE<sup>-/-</sup> mice (6, 15 weeks old). Previously, guantitative PCR studies demonstrated that ghrelin is expressed in whole kidney [9, 42]. In particular, Jeffrey et al. [42] indicated that they were unable to detect significant protein expression in glomeruli even if mRNA expression was seen in other studies [9, 43]. Yabuki et al., [43] had detected a ghrelin peptide with a specific band of 13 KDa in mouse kidney. The molecular weight of this protein matched that of prepro-ghrelin that was seen by Jeffrey et al. [42]. These findings are important because they support our data in control mice; at the same time, they are strengthened from our findings found in ApoE-/mice. Taken together all data we can underline the presence of ghrelin in the kidney.

From physiological point of view, we can explain our results considering that ghrelin is able to induce contractile response that is relevant in renal arteries [44]. In fact, Dimitrova et al. [44] suggested that ghrelin exerts its contractile activity on mammalian renal arteries by augmenting endothelin-1 triggered intracellular signaling in smooth muscular cells, and/or by stimulating the endothelial release of some mediator playing a contractile action. These mediators could be different from catecholamines, angiotensin II, superoxide anions and cyclooxygenase-derived prostanoids.

Therefore, we considered the possibility that ghrelin could have a role in protecting against

aging-related diseases through a multitasking protein like SIRT1. In particular, SIRT1 acts as a cardioprotective molecule that protects the heart from aging and ischemia/reperfusion injury, resists hypertrophic and oxidative stresses, inhibits cardiomyocytes apoptosis, and regulates cardiac energy metabolism [45-48]. Moreover, it is a survival

factor involved in lifespan extension as discussed by Ryu et al. [49]. Thus, we evaluated SIRT1 expression in all experimental groups and observed a decreased in SIRT1 positivity associated to aging in control animals as well as to pathological alterations in young and old ApoE<sup>-/-</sup> mice. At our knowledge, few are the papers stating that the link between ghrelin and SIRT1 is found not only in kidney but also in different organs; they have all been mentioned above. Consequently, to better stress the action of ghrelin-SIRT1 system, we have carried out this study, the results of which have demonstrated that both proteins are localized in the same structures of the kidney, even if sometimes they showed different positivity. These results are consistent with the data obtained by Yang et al. in both in vivo and in vitro experiments, which confirmed the existence of a ghrelin-SIRT1 system that plays a role in sodium fluid homeostasis [16]. These Authors suggested that increased expression of SIRT1 occurred through activation of the ghrelin pathway in low-salt diet rats. This finding support or results indicating the presence of these proteins in the kidney.

Last but not less important than other findings is the relationship we observed between SIRT1 and AQP1 and 2. In this regard, we studied the positivity of AQP1 and 2, showing that they have the same positivity pattern as other proteins studied. Interestingly, AQP1 and 2 decreased in old control animals and in ApoE<sup>-/-</sup> mice at different ages, and they co-localized partially with SIRT1 and ghrelin. Moreover, it is important to remember that AQP1 was present in cortex and medulla, whereas AQP2 was only evident in medulla. This pattern can be discussed considering the functions of these proteins and their relationship with SIRT1 and ghrelin. It is known that AQP1 is essential for



**Figure 6.** A schematic representation of the ghrelin-mediated pathway. Immunohistochemical analysis confirms the role of ghrelin-SIRT1 system, both in renal cortex and medulla. The results show a correlation also among ghrelin, SIRT1 and AQP1 in renal cortex and among ghrelin, SIRT1, AQP1 and 2 in renal medulla. This complex interaction is characterized by changes that occur with aging and in ApoE<sup>-/-</sup> mice leading to renal injuries. Consequently, achieving the steady psychological state of ghrelin concentration could be a new therapeutic approach to combat age-related renal dysfunctions. (-): negative, (+/-): very weak, (+): weak, (++): moderate, (+++): strong positivity.

constitutive water reabsorption in proximal tubules and Henle loops [50]; this is confirmed also in AQP1 gene knockout mice, which have a severe urinary concentration defect [51]. Furthermore, in mice lacking AQP1, tubules showed an 80% and a 90% reduction in osmotic water permeability in proximal tubules and in Henle loops respectively. As regards AOP2, many studies have underlined its essential role in regulating body water balance mediated by vasopressin [50]. Moreover, AQP2 in collecting ducts is involved not only in water movement, but also in cell migration and epithelial morphogenesis [52, 53]. It has been shown that it decreases significantly in collecting ducts in rats with cisplatin-induced kidney injury determined by ischemia/reperfusion disease or sepsis [54]. Conversely, AQP2 mRNA expression was significantly up-regulated in the kidneys of cirrhotic rats [55]. These findings imply that AQP2 plays different roles in several renalrelated diseases. Moreover, they suggest that SIRT1 overexpression increases AQP2 expression [54]. These results agree with our results, proving the crucial role of SIRT1 in AQP2 regulation. In fact, SIRT1 decreases in old control groups and when its positivity is already low in young animal models, such as ApoE<sup>-/-</sup> mice, the animals show morphological renal alterations.

In conclusion, we propose not only that ghrelin-SIRT1 is a system, but also that it is linked to AQP proteins and that together constitute a complex axis for normal renal functions. When this axis does not operate correctly, significant changes are found in the structures and physiology of the kidney, related to aging with or without comorbidities. **Figure 6** shows the relationship among ghrelin-SIRT1-AQP1 and 2. These findings suggest that achieving a steady physiological state of ghrelin concentrations could be a candidate therapeutic approach to combat age-related renal metabolic and physiological changes.

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#### Disclosure of conflict of interest

#### None.

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