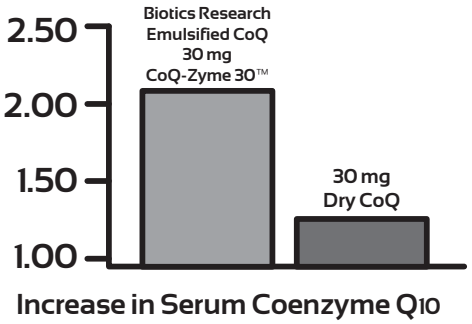
# CoQ-Zyme 30™ and CoQ-Zyme 100 Plus™


**CoQ-Zyme 30™** and **CoQ-Zyme 100 Plus™** each supply a highly bioavailable dose of ubiquinone coenzyme Q10 (CoQ10) due to the proprietary emulsification process used for delivery. In a double-blind clinical study, daily ingestion of 1 tablet (30 mg) of Biotics Research Corporation's emulsified CoQ10 for 4 weeks was shown to increase plasma CoQ10 levels by 210%, equivalent to 90-100 mg of dry CoQ10. Furthermore, dry CoQ10 powder increased serum levels in only 57% of subjects, while the Biotics Research emulsified CoQ10 produced an increase in serum CoQ10 levels in 80% of the subjects.[1,2]

Importantly, Biotics Research uses no soy byproducts, no artificial flavors or colorants, no propylene glycol, and no detergents or other artificial surfactants in our proprietary emulsification process.




CoQ10 is a fat-soluble, high molecular weight compound produced by the body for the basic functioning of cells. As a cellular component, CoQ10 has two primary functions in the body; first, to act in the transfer of electrons as a necessary part of ATP production, and second, to function as an essential antioxidant. In the body, CoQ10 is ubiquitous in all cells (thus its name "ubiquinone"); however, in humans, the highest concentrations are found in the heart, liver, muscle, kidney and brain.

## Nutritional Support for Energy Production

A necessary component of ATP production, CoQ10 plays a critical role in mitochondrial respiration. In addition to intra-mitochondrial processes, it plays a vital role in extra-mitochondrial processes, including its regulatory action in the NADH oxidoreductase (complex I) function of the plasma membrane [3,4] as well as its function in the redox potential of both the Golgi complex and the plasma membrane.[5,6] Consequently, its role is vital to the cellular energy generating systems.[7] Often termed "the hub around which life processes revolve in the human body", CoQ10 participates in all energy processes. As the only lipid-soluble antioxidant synthesized endogenously,[8] its absence or inadequate supply results in diminished energy production and suboptimal cellular function.

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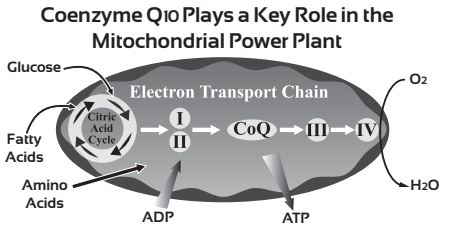


## CoQ10 and Cardiac Health

Over eighty drugs, the most notable being statin drugs, are known to have a negative impact on the body's ability to produce CoQ10.[9] Statins block the enzyme HMG-CoA reductase, which is a functional component in the body's ability to synthesize CoQ10. With statin therapy, reductions in CoQ10 between 16-54% have been documented.[10] As a result, there is often a need to increase the daily intake of CoQ10.

CoQ10 supplementation has demonstrated numerous cardiovascular benefits via its action to increase both myocardial and cardiac mitochondrial competence, as well as 'myocardial tolerance' towards the stress of hypoxia/reoxygenation.[11] Because the cells of the heart have a high dependence on ATP, they contain a large quantity of mitochondria, which support both the contractile role and the cardiac output.[12] Therefore, a depleted supply of CoQ10 may result in a negative impact on mitochondrial energy,[13] and supplemental CoQ10, therefore, exerts a positive influence on the performance of the heart.[14] In addition, CoQ10 is also speculated to improve the integrity of the vascular tissue indirectly via its inhibition of oxidative damage to LDL.[15]

## CoQ10 as an Antioxidant

Exogenous CoQ10 has been shown to protect cells against oxidative stress,[16] as well as to improve arterial endothelial function of the peripheral circulation in patients with Type II diabetes and dyslipidemia.[17] The body readily converts CoQ10 (ubiquinone) to the reduced form, ubiquinol, which predominates when there is a net generation of ATP in the cell. In addition to improving oxidation via its production of high energy phosphates, and as a consequence to its free radical scavenging activities,[18] CoQ10 functions as a potent intracellular antioxidant, and possesses powerful activity against free radical species.[19] Numerous studies have demonstrated the antioxidant benefits of CoQ10 supplementation, including its role in reducing the level of mitochondrial reactive oxygen species and decreasing DNA damage.[20]

## CoQ10 and Immune Function

Because cells and tissues involved in immune function are highly dependent upon energy, they require an adequate supply of CoQ10. In studies with elderly animals, immune function is shown to decline with age. In these studies, a suppression of the immune response was associated with a marked decline in CoQ10 levels in thymic tissue.[21] Studies have also demonstrated an immune-supportive role with the use of oral CoQ10. For example, a suboptimal concentration of CoQ10 has been observed in asthmatic patients. They postulated this low level of CoQ10 yielded an antioxidant imbalance, which increased the incidence of asthma.[22] In another study, corticosteroid-dependent bronchial asthmatic patients exhibited a decrease in CoQ10 levels and CoQ10 supplementation resulted in a reduced corticosteroid requirement for symptom relief.[23]

In addition, oral administration of CoQ10 has been shown to enhance the phagocytic activity of macrophages, and to increase the proliferation of granulocytes in response to infection.[21]

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## References


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2. Stiles J, Sparks B, Klenda B, Pillors M, Bucci L. Enhanced uptake in humans of coenzyme Q10 from an emulsified form. Third International Congress of Biomedical Gerontology. June, 1989.

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## CoQ-Zyme 100 Plus™

Each capsule of CoQ-Zyme 100 Plus™ supplies 100 mg of emulsified CoQ10, as well as a full complement of important B vitamins. Each capsule also supplies 100% of the Daily Value of B-complex vitamins, along with 80 mcg each of SOD and Catalase from our exclusive Vegetable Culture.

## CoQ-Zyme 30™

Each tablet of CoQ-Zyme 30™ supplies 30 mg of emulsified CoQ10, as well as 30 mcg each of Superoxide Dismutase (SOD) and Catalase, key antioxidant enzymes from our exclusive Vegetable Culture.

**CoQ-Zyme 100 Plus™** is available in 60 count bottles (#2617)

### Supplement Facts

**Serving Size: 1 Capsule**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Thiamin (B1) (as cocarboxylase chloride) | 1.5 mg | 125% |
| Riboflavin (B2) (as riboflavin-5-phosphate) | 1.7 mg | 131% |
| Niacin (as niacin & niacinamide) | 20 mg | 125% |
| Vitamin B6 (as pyridoxal-5-phosphate) | 2 mg | 118% |
| Folate (as calcium folinate) | 400 mcg DFE | 100% |
| Vitamin B12 (as methylcobalamin) | 6 mcg | 250% |
| Biotin | 300 mcg | 1,000% |
| Pantothenic acid (as calcium pantothenate) | 10 mg | 200% |
| Coenzyme Q10 (emulsified) | 100 mg | * |
| Superoxide Dismutase (from vegetable culture†) | 80 mcg | * |
| Catalase (from vegetable culture†) | 80 mcg | * |

*** Daily Value not established**

**Other ingredients:** Capsule shell (gelatin and water), gum arabic and magnesium stearate (vegetable source).

† Specially grown, biologically active vegetable culture containing naturally associated phytochemicals including polyphenolic compounds with SOD and catalase, dehydrated at low temperature to preserve associated enzyme factors.

**This product is gluten, dairy and GMO free.**

**RECOMMENDATION:** One (1) capsule each day as a dietary supplement or as otherwise directed by a healthcare professional.

**CAUTION:** Not recommended for pregnant or lactating women.

**KEEP OUT OF REACH OF CHILDREN**
Store in a cool, dry area.
Sealed with an imprinted safety seal for your protection.

Product # 2617 Rev. 07/18

**CoQ-Zyme 30™** is available in a 60-count bottle (#2616)

### Supplement Facts

**Serving Size: 1 Tablet**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Coenzyme Q10 (emulsified) | 30 mg | * |
| Superoxide Dismutase (from vegetable culture †) | 30 mcg | * |
| Catalase (from vegetable culture †) | 30 mcg | * |

*** Daily Value not established**

**Other ingredients:** Cellulose, stearic acid (vegetable source), gum arabic, magnesium stearate (vegetable source), modified cellulose gum, silica and food glaze.

† Specially grown, biologically active vegetable culture containing naturally occuring and/or organically bound phytochemicals including polyphenolic compounds with SOD and catalase, dehydrated at low temperature to preserve associated enzyme factors.

**This product is gluten, dairy and GMO free.**

**RECOMMENDATION:** One (1) tablet each day as a dietary supplement or as otherwise directed by a healthcare professional.

**KEEP OUT OF REACH OF CHILDREN**
Store in a cool, dry area.
Sealed with an imprinted safety seal for your protection.

Product # 2616 Rev. 09/18

To place your order for **CoQ-Zyme 100 Plus™ or CoQ-Zyme 30™** please contact us below.

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**Biotics Research Corporation**
is pleased to announce a ***new product:***

# CoQ-Zyme 100 Plus™

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Biotics Research is pleased to announce a new product, **CoQ-Zyme 100 Plus™**. Each capsule of **CoQ-Zyme 100 Plus™** supplies 100 mg of emulsified coenzyme Q10, as well as a full complement of important B vitamins, with each capsule supplying 100% of the Daily Value of B-complex vitamins, along with 80 mcg each of SOD and Catalase from our exclusive Vegetable Culture.

Based on a double blind clinical study, daily ingestion of 1 tablet (30 mg) of Biotics Research Corporation's emulsified CoQ10 for 4 weeks was demonstrated to increase plasma CoQ10 levels by 210%, equivalent to 90-100 mg of dry CoQ10. Furthermore, dry CoQ10 powder increased serum levels in only 57% of subjects, while the Biotics Research Corporation emulsified CoQ10 produced an increase in serum CoQ10 levels in 80% of the subjects.[1,2] Importantly, Biotics Research uses no soy byproducts, no artificial flavors or colorants, no propylene glycol, and no detergents or other artificial surfactants in our proprietary emulsification process.


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**Biotics Research Corporation**
is pleased to announce a ***new product:***

# CoQ-Zyme 100 Plus™

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## CoQ-Zyme 100 Plus™

Product Code: #2617
Suggested Retail Price: $52.50
60 Capsules

Dosage: 1 Capsule

### Supplement Facts

**Serving Size: 1 Capsule**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Thiamin ($B_1$) (as cocarboxylase chloride) | 1.5 mg | 100% |
| Riboflavin ($B_2$) (as riboflavin-5-phosphate) | 1.7 mg | 100% |
| Niacin (as niacin & niacinamide) | 20 mg | 100% |
| Vitamin $B_6$ (as pyridoxal-5-phosphate) | 2 mg | 100% |
| Folate (as calcium folinate) | 400 mcg | 100% |
| Vitamin $B_{12}$ (as methylcobalamin) | 6 mcg | 100% |
| Biotin | 300 mcg | 100% |
| Pantothenic acid (as calcium pantothenate) | 10 mg | 100% |
| Coenzyme Q10 (emulsified) | 100 mg | * |
| Superoxide Dismutase (from vegetable culture†) | 80 mcg | * |
| Catalase (from vegetable culture†) | 80 mcg | * |

*** Daily Value not established**

**Other ingredients:** Capsule shell (gelatin and water), gum arabic and magnesium stearate (vegetable source).

† Specially grown, biologically active vegetable culture containing naturally associated phytochemicals including polyphenolic compounds with SOD and catalase, dehydrated at low temperature to preserve associated enzyme factors.

**RECOMMENDATION:** One (1) capsule each day as a dietary supplement or as otherwise directed by a healthcare professional.

**Caution:** Not recommended for pregnant women.

**KEEP OUT OF REACH OF CHILDREN**
Store in a cool, dry area.
Sealed with an imprinted safety seal for your protection.

NDC#55146-02617 Rev. 07/11



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# Other Primary Cardiometabolic Support Formulas


## Berberine HCl

**Product Number: 5212 (90C)**

Berberine HCl supplies Berberine HCl isolated from Berberis vulgaris (barberry). Berberine has a long history of use in both Chinese and Ayurvedic medicine to support normal glucose and/or lipid metabolism.

**Active Ingredients:**

Berberine Hydrochloride. Berberine HCl is purified from Berberis vulgaris.

**Supplement Facts**

**Serving Size: 1 Capsule**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Berberine Hydrochloride | 500 mg | * |

*** Daily Value not established**

**Other ingredients:** Cellulose, capsule shell (gelatin and water) and magnesium stearate (vegetable source).

**Berberine HCl is purified from Berberis vulgaris.**

**This product is gluten, dairy and GMO free.**


## Biomega-*1000*™

**Product Number: 1417 (90C)**

**Biomega-*1000*™** provides a potent dose of omega-3 essential fatty acids (EFAs), sourced from a strategically-placed facility in the far South Pacific Ocean off the coast of Chile, guaranteeing the freshest raw material available, full traceability of the product and unparalleled purity. **Biomega-*1000*™** capsules deliver an impressive 1,000 mg of EPA and DHA, making it the ideal choice for therapeutic dosing.

**Active Ingredients:**

Vitamin E (as mixed tocopherol), Omega-3 fatty acids

**Supplement Facts**

**Serving Size: 1 Softgel Capsule**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Calories | 15 | |
| Calories from Fat | 15 | |
| Total Fat | 1.5 g | 2%* |
| Saturated Fat | <1 g | <3%* |
| Vitamin E (as mixed tocopherols) | 4 mg | 27% |
| Omega-3 fatty acids | 1.14 g | † |

*** Percent Daily Values based on a 2,000 calorie diet**

**† Daily Value not established**

**Other Ingredients:** Capsule shell (gelatin, glycerin, water and carob).

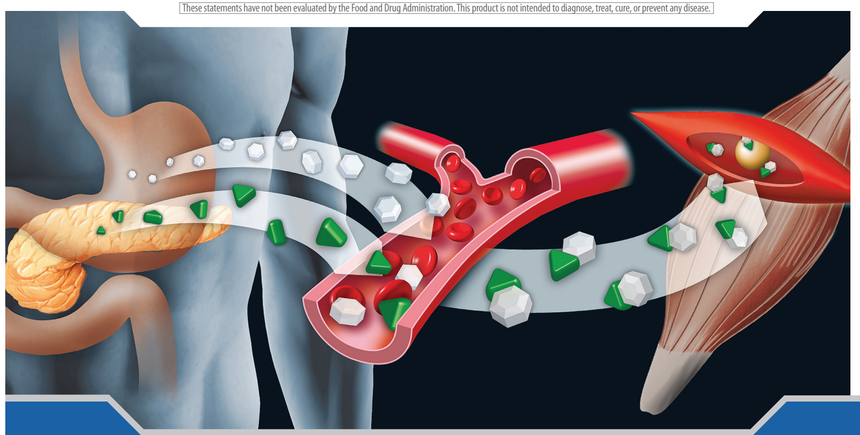
**Contains ingredients derived from Anchovy.**

**This product is gluten and dairy free.**

Each softgel capsule of **Biomega-*1000*™** contains 1,000 mg of EPA and DHA, providing a natural source of the following total Omega-3 fatty acids:

| | |
|---|---|
| EPA (Eicosapentaenoic acid) | 570 mg |
| DHA (Docosehexaenoic acid) | 430 mg |
| Additional Omega-3 Fatty Acids | 140 mg |

These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.




## CoQ-Zyme 100 Plus™

**Product Number: 2617 (60C)**

**CoQ-Zyme 100 Plus™** supplies 100 mg of emulsified co-enzyme Q10, along with the B-complex vitamins Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Biotin and Pantothenic acid.

**Active Ingredients:**

Thiamin (B1) (as cocarboxylase chloride); Riboflavin (B2) (as riboflavin-5-phosphate); Niacin (as niacin & niacinamide); Vitamin B6 (as pyridoxal-5-phosphate); Folate (as calcium folinate); Vitamin B12 (as methylcobalamin); Biotin; Pantothenic acid (as calcium pantothenate); Coenzyme Q10 (emulsified); Superoxide Dismutase (from vegetable culture†); Catalase (from vegetable culture†) † Specially grown, biologically active vegetable culture containing naturally associated phytochemicals including polyphenolic compounds with SOD and catalase, dehydrated at low temperature to preserve associated enzyme factors.

**Supplement Facts**

**Serving Size: 1 Capsule**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Thiamin (B1) (as cocarboxylase chloride) | 1.5 mg | 100% |
| Riboflavin (B2) (as riboflavin-5-phosphate) | 1.7 mg | 100% |
| Niacin (as niacin & niacinamide) | 20 mg | 100% |
| Vitamin B6 (as pyridoxal-5-phosphate) | 2 mg | 100% |
| Folate (as calcium folinate) | 400 mcg | 100% |
| Vitamin B12 (as methylcobalamin) | 6 mcg | 100% |
| Biotin | 300 mcg | 100% |
| Pantothenic acid (as calcium pantothenate) | 10 mg | 100% |
| Coenzyme Q10 (emulsified) | 100 mg | * |
| Superoxide Dismutase (from vegetable culture†) | 80 mg | * |
| Catalase (from vegetable culture†) | 80 mcg | * |

*** Daily Value not established**

**Other ingredients:** Capsule shell (gelatin and water), gum arabic and magnesium stearate (vegetable source).


## GlucoBalance®

**Product Number: 1850 (90C) and 1851 (180C)**

**GlucoBalance®** was formulated by Johnathan Wright, MD and Alan Gaby, MD, and is specifically targeted to support individuals with undesirable blood sugar levels. In these individuals, specific dietary needs may exist, necessitating the need for higher amounts of important micronutrients. Deficiencies of certain minerals such as potassium, magnesium, zinc and chromium, are well documented in those with uncontrolled hyperglycemia, and may predispose these individuals to carbohydrate intolerance. {Chehade JM, Sheikh-Ali M, Mooradian AD. The Role of Micronutrients in Managing Diabetes. Diabetes Spectrum. September 21, 2009 22:4 214-218.} **GlucoBalance®** may also be beneficial in supporting normal, healthy blood lipid levels.

**Active Ingredients:**

Vitamin A (as retinyl acetate); Vitamin C (as calcium ascorbate and ascorbic acid); Vitamin D (as cholecalciferol); Vitamin E (as d-alpha tocopheryl acetate); Thiamin (B1) (as thiamin mononitrate); Riboflavin (B2); Niacin (as niacinamide and niacin); Vitamin B6 (as pyridoxine hydrochloride); Folate (as calcium folinate); Vitamin B12 (as methylcobalamin); Biotin; Pantothenic Acid (as calcium pantothenate); Calcium (as ascorbate, citrate and carbonate); Magnesium (as aspartate, citrate and oxide); Zinc (as zinc picolinate and zinc citrate); Selenium (as selenomethionine); Copper (as copper gluconate); Manganese (as manganese citrate); Chromium (as chromium aspartate); Potassium (as potassium aspartate); Vanadium (as vanadium aspartate); L-Carnitine fumarate

**Supplement Facts**

**Serving Size: 6 Capsules**

**Servings Per Container: 15**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Vitamin A (as retinyl acetate) | 1,500 mcg RAE | 167% |
| Vitamin C (as calcium ascorbate and ascorbic acid) | 500 mg | 556% |
| Vitamin D (as cholecalciferol) | 2.5 mcg | 13% |
| Vitamin E (as d-alpha tocopheryl acetate) | 270 mg | 1,800% |
| Thiamin (B1) (as thiamin mononitrate) | 50 mg | 4,167% |
| Riboflavin (B2) | 25 mg | 1,923% |
| Niacin (as niacinamide and niacin) | 150 mg | 938% |
| Vitamin B6 (as pyridoxine hydrochloride) | 30 mg | 1,765% |
| Folate (as calcium folinate) | 800 mcg DFE | 200% |
| Vitamin B12 (as methylcobalamin) | 50 mcg | 2,083% |
| Biotin | 3,000 mcg | 10,000% |
| Pantothenic Acid (as calcium pantothenate) | 100 mg | 2,000% |
| Calcium (as ascorbate, citrate and carbonate) | 200 mg | 15% |
| Magnesium (as aspartate, citrate and oxide) | 400 mg | 95% |

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Zinc (as zinc picolinate and zinc citrate) | 30 mg | 272% |
| Selenium (as selenomethionine) | 150 mcg | 272% |
| Copper (as copper gluconate) | 2 mg | 222% |
| Manganese (as manganese citrate) | 20 mg | 870% |
| Chromium (as chromium aspartate) | 1,000 mcg | 2,857% |
| Potassium (as potassium aspartate) | 99 mg | 2% |
| Vanadium (as vanadium aspartate) | 20 mcg | * |
| L-Carnitine fumarate | 30 mg | * |

*** Daily Value not established**

**Other ingredients:** Capsule shell (gelatin and water) and magnesium stearate (vegetable source).

**This product is gluten, dairy and GMO free.**

These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.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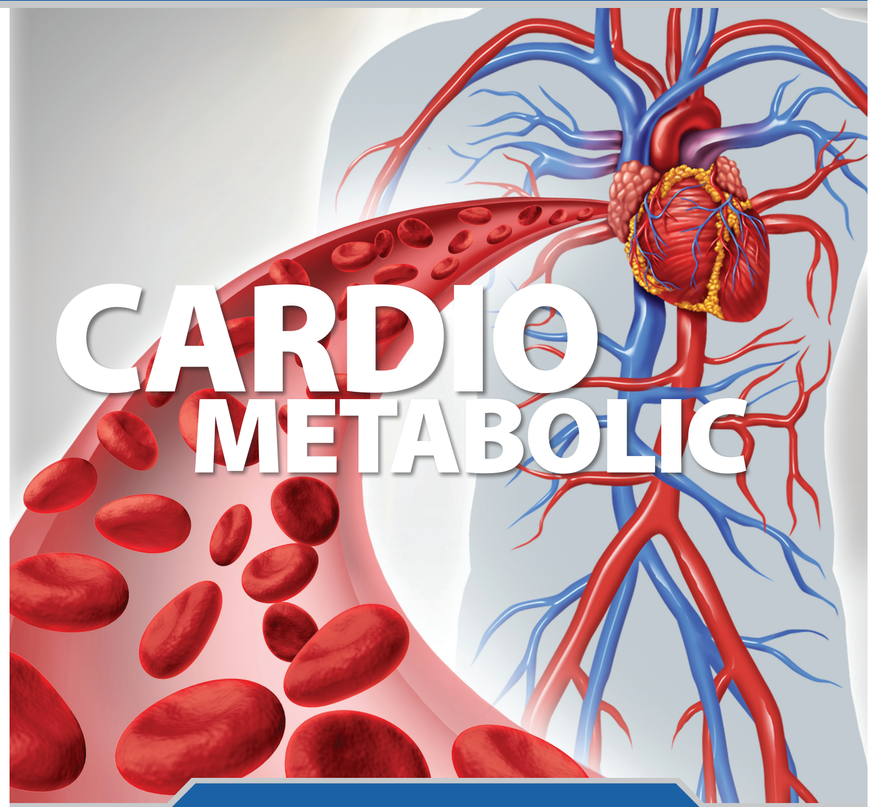
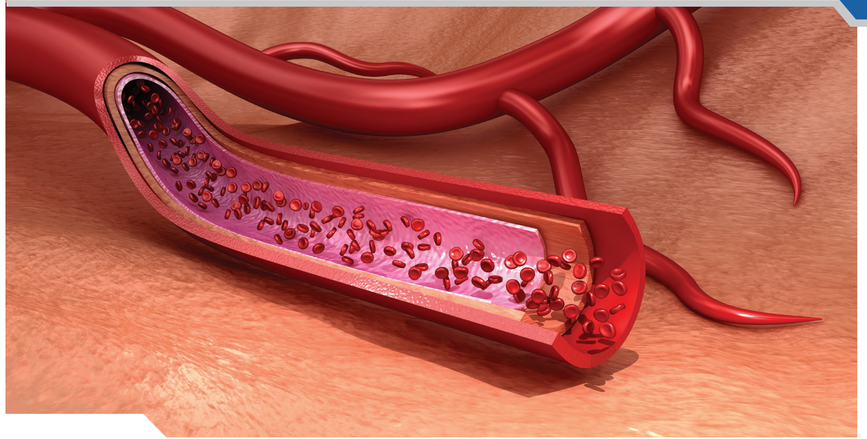



BIOTICS RESEARCH CORPORATION

**SUPPLEMENTS TO SUPPORT CARDIOVASCULAR & METABOLIC HEALTH**

# Cardiometabolic Support from Biotics Research



Cardiovascular disease (CVD) remains the leading cause of mortality for both men and women worldwide. Cardiometabolic health involves a group of interrelated factors and encompasses healthy blood lipid levels, circulatory and blood vessel function, optimal blood sugar levels and overall heart health.

With a growing public concern for cardiometabolic health, Biotics Research Corporation, in conjunction with Dr. Mark Houston, Associate Clinical Professor of Medicine at Vanderbilt Medical School and Director of Hypertension Institute and Vascular Biology in Nashville, has developed a unique selection of "heart-healthy" nutritional formulas.

Adjunct Products:
Bio-D-Mulsion Forte®, Bio-K-Forte Caps®, CurcumRx®,
Mg-Zyme™, and Optimal EFAs® Caps




## Bio-CardioSirt BP®

**Product Number: 2905 (8oz)**

**Bio-CardioSirt BP®** supplies a unique, patented (US 9,642,885 B2) combination of seven (7) key micronutrients that support normal, healthy blood pressure levels. These include the vitamins C, D and B6, along with Biotin, Magnesium, Taurine and Grape Seed extract.

**Active Ingredients:**
Vitamin C (as magnesium ascorbate), Vitamin D (as cholecalciferol), Vitamin B6 (as pyridoxine hydrochloride), Biotin, Magnesium (as magnesium ascorbate), Taurine and MegaNatural®-BP grape seed extract. MegaNatural®-BP and its logo are trademarks of Constellation Brands, Inc.

**Supplement Facts**
**Serving Size: 1 rounded scoop (approx. 7.8 g)**
**Servings Per Container: 30**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Vitamin C (as magnesium ascorbate) | 1,000 mg | 1666% |
| Vitamin D (as cholecalciferol) | 2,000 IU | 500% |
| Vitamin B6 (as pyridoxine hydrochloride) | 100 mg | 5000% |
| Biotin | 2,000 mcg | 667% |
| Magnesium (as magnesium ascorbate) | 87 mg | 22% |
| Proprietary Blend | 6,150 mg | |
| Taurine | | * |
| MegaNatural®-BP grape seed extract | | * |

***Daily Value not established**

MegaNatural®-BP and its logo are trademarks of Constellation Brands Inc.


## EFA-Sirt Supreme®

**Product Number: 1412 (180C)**

**EFA-Sirt Supreme®** supplies a unique, highly concentrated essential fatty acid blend, providing an extremely effective relative combination of EPA, DHA and GLA, with all-natural mixed tocopherols, specially formulated to be high in gamma (γ)-Tocopherol.

**Active Ingredients:**
Natural Mixed Tocopherols including, d-gamma tocopherols, d-delta tocopherol, d-beta tocopherol, and d-alpha tocopherol, 360 mg; GLA (Gamma Linolenic Acid) 750 mg; Total Omega-3 Fatty Acid 1.7 g; EPA (Eicosapentaenoic Acid) 900 mg; DHA (Docosahexaenoic Acid) 600 mg; Additional Omega-3 Fatty Acids 200 mg

**Supplement Facts**
**Serving Size: 6 Softgel Capsules**
**Servings Per Container: 30**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Calories (energy) | 70 | |
| Total Fat | 7 g | 9%† |
| Saturated Fat (not more than) | 1 g | 5%† |
| Cholesterol | 5 mg | 2%† |
| Total Carbohydrate | <1 g | <1%† |
| Protein | 2 g | |
| Natural Mixed Tocopherols including d-gamma tocopherols, d-delta tocopherol d-beta tocopherol, and d-alpha tocopherol | 360 mg | * |
| GLA (Gamma Linolenic Acid) | 750 mg | * |
| Total Omega-3 Fatty Acid | 1.7 g | * |
| EPA (Eicosapentaenoic Acid) | 900 mg | * |
| DHA (Docosahexaenoic Acid) | 600 mg | * |
| Additional Omega-3 Fatty Acids | 200 mg | * |


## Lipid-Sirt®

**Product Number: 2935 (240C)**

**Lipid-Sirt®** provides support for vascular integrity and healthy aging by supporting and stimulating sirtuin activity. Specific nutritional compounds have been demonstrated to have a positive impact on cholesterol levels. **Lipid-Sirt®** supplies some of these specific nutrients, which have been demonstrated to: 1) modify the production of cholesterol in the liver by reacting with hepatic enzymes, 2) increase cholesterol excretion via the bile, and 3) inhibit cholesterol uptake from the intestine.

**Active Ingredients:**
Pantethine, Plant Sterols (from soybean), Green Tea Extract (50% EGCG (leaf), Delta-tocotrienol (from annatto seed), Phytolens®** (Lens esculenta extract) (husk). Contains ingredients derived from soybean. ** Phytolens® is a registered trademark of Biotics Research Corporation, U.S. Patent No. 5,762,936.

**Supplement Facts**
**Serving Size: 4 Capsules**
**Servings Per Container: 60**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Pantethine | 450 mg | * |
| Plant Sterols (from soybean) | 400 mg | * |
| Green Tea Extract (50% EGCG) (leaf) | 300 mg | * |
| Delta-tocotrienol (from annatto seed) | 37.5 mg | * |
| Phytolens®** (Lens esculenta extract) (husk) | 2.5 mg | * |

***Daily Value not established**

**Other ingredients:** Capsule shell (gelatin and water), cellulose, silica, magnesium stearate (vegetable source), and modified cellulose gum.

**Contains ingredients derived from soybean.**

** **Phytolens®** is a registered trademark of Biotics Research Corporation. U.S. Patent No. 5,762,936

**This product is gluten and dairy free.**






## Red Yeast Rice

**Product Number: 8000 (90C)**

The yeast *Monascus purpureus*, grown on rice, is known in the nutritional industry as **Red Yeast Rice** (RYR). In many Asian countries, it is a dietary staple. In Traditional Chinese Medicine (TCM), **Red Yeast Rice** has been utilized for thousands of years. In addition to its functional impact on blood lipids, it has been implicated in the support of digestion, blood movement, and the strengthening of the spleen. To ensure safety, Biotics Research's **Red Yeast Rice** is screened for the mycotoxin, citrin.

**Active Ingredients:**
Red Yeast Rice

**Supplement Facts**
**Serving Size: 3 Capsules**
**Servings Per Container: 30**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Calories | 10 | |
| Total Carbohydrates | 2 g | <1%* |
| Red Yeast Rice | 2,400 mg | † |

**† Daily Value not established**
*** Percent Daily Values are based on a 2,000 calorie diet**

**Other ingredients:** Capsule shell (gelatin and water), and magnesium stearate (vegetable source).

**This product is gluten and dairy free.**


## ResveraSirt-HP®

**Product Number: 2930 (30C) and 2931 (120C)**

Each capsule of **ResveraSirt-HP®** supplies 250 mg of purified Trans-Resveratrol, produced via a proprietary fermentation process, free from contaminents such as benzopyrine, often seen in other commonly used and less costly sources of Resveratrol. Quercetin is added for its ability to slow the metabolism of resveratrol. Also included in this formula is IP6 (phytic acid or phytin), a 6-phosphate ester of inositol derived from rice. Effective at a wide pH range, IP6 is a strong metal chelator, and thus aids in stabilizing the formula. By using the purified form of Resveratrol, **ResveraSirt-HP®** does not cause the GI distress often associated with lesser grades of this material.

**Active Ingredients:**
Trans-Resveratrol (from fermentation), Quercetin, Calcium magnesium phytate

**Supplement Facts**
**Serving Size: 1 Capsule**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Trans-Resveratrol (from fermentation) | 250 mg | * |
| Quercetin | 25 mg | * |
| Calcium magnesium phytate | 25 mg | * |

*** Daily Value not established**

**Other ingredients:** Microcrystalline cellulose and capsule shell (gelatin and water).

**This product is gluten and dairy free.**


## VasculoSirt®

**Product Number: 2924 (150C) and 2925 (300C)**

**VasculoSirt®** is a revolutionary nutritional supplement designed to slow vascular aging, promote vascular and heart health, and provide healthy support for blood pressure, cholesterol, glucose and insulin levels.

**Active Ingredients:**
Vitamin A (as mixed carotenoids), Vitamin C (as ascorbic acid), Vitamin D3 (as cholecalciferol), Vitamin K (as menaquinone-7 (extract of Bacillus subtilis natto) and as phytonadione), Thiamin (B1) (as thiamin mononitrate), Riboflavin (B2), Niacin, Vitamin B6 (as pyridoxine HCl), Folate (as calcium folinate) Vitamin B12 (as methylcobalamin), Biotin, Pantothenic Acid (as calcium pantothenate), Magnesium (as magnesium glycinate*), Zinc (as zinc picolinate), Selenium (as selenomethionine), Copper (as copper citrate), Coenzyme Q10 (emulsified), Trans-Resveratrol (from fermentation), R-Alpha Lipoic Acid (from stabilized sodium salt), Green Tea Extract (50% EGCG) (leaf), Acetyl-L-Carnitine hydrochloride, Olive Extract (Olea europaea) (fruit), Quercetin (Dimorphandra mollis), Ginkgo Extract (Ginkgo biloba) (leaf), Phytolens*** (Lens esculenta extract), Lutien (from Aztec Marigold flower), Lycopene (from Tomato). *Albion® brand Magnesium Glycinate. Albion laboratories, Inc. of Clearfield, Utah. **Phytolens® is a registered trademark of Biotics Research Corporation. US. Patent No. 5,762,936, Biotics Research Corporation.

**Supplement Facts**
**Serving Size: 5 Capsules** — **Servings Per Container: 30**

| | Amount Per Serving | % Daily Value |
|---|---|---|
| Vitamin A (as mixed carotenoids) | 1,750 mcg | 35% |
| Vitamin C (as ascorbic acid) | 250 mg | 417% |
| Vitamin D (as cholecalciferol) | 2,000 IU | 500% |
| Vitamin K (as Beta-Q™ menaquinone-7 (extract of Bacillus subtilis natto) and as phytonadione) | 50 mcg | 63% |
| Thiamin (B1) (as thiamin mononitrate) | 5 mg | 333% |
| Riboflavin (B2) | 5 mg | 294% |
| Niacin | 20 mg | 100% |
| Vitamin B6 (as pyridoxine HCl) | 50 mg | 2,500% |
| Folate (as calcium folinate) | 667 mcg | 167% |
| Vitamin B12 (as methylcobalamin) | 500 mcg | 833% |
| Biotin | 150 mcg | 50% |
| Pantothenic Acid (as calcium pantothenate) | 125 mg | 1,250% |
| Magnesium (as magnesium glycinate*) | 100 mg | 25% |
| Zinc (as zinc picolinate) | 15 mg | 100% |
| Selenium (as selenomethionine) | 50 mcg | 91% |
| Copper (as copper citrate) | 0.5 mg | 28% |
| Coenzyme Q10 (emulsified) | 50 mg | † |
| Trans-Resveratrol (from fermentation) | 50 mg | † |
| R-Alpha Lipoic Acid (from stabilized sodium salt) | 50 mg | † |
| Green Tea Extract (50% EGCG) (leaf) | 500 mg | † |
| Acetyl-L-Carnitine hydrochloride | 500 mg | † |
| Olive Extract (Olea europaea) (fruit) | 50 mg | † |
| Quercetin (Dimorphandra mollis) | 50 mg | † |
| Ginkgo Extract (Ginkgo biloba) (leaf) | 50 mg | † |
| Phytolens*** (Lens esculenta extract) (husk) | 5 mg | † |
| Lutein (from Tagetes erecta (flower)) | 5 mg | † |
| Lycopene (from Tomato) | 5 mg | † |

**† Daily Value not established**

**Other ingredients:** Capsule shell (gelatin and water), magnesium stearate (vegetable source), and silica.

*** Albion®** is the trademark of Albion laboratories, Inc. of Clearfield, Utah.

**** MenaQ7®** is the trademark of NattoPharma, Norway. Patents pending.

***** Albion®** brand Magnesium Glycinate. Albion laboratories, Inc. of Clearfield, Utah.

****** Phytolens®** is a registered trademark of Biotics Research Corporation US. Patent No. 5,762,936. Biotics Research Corporation.

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# $CoQ_{10}$ supplementation rescues nephrotic syndrome through normalization of $H_2S$ oxidation pathway

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ABSTRACT

Nephrotic syndrome (NS), a frequent chronic kidney disease in children and young adults, is the most common phenotype associated with primary coenzyme $Q_{10}$ ($CoQ_{10}$) deficiency and is very responsive to $CoQ_{10}$ supplementation, although the pathomechanism is not clear. Here, using a mouse model of CoQ deficiency-associated NS, we show that long-term oral $CoQ_{10}$ supplementation prevents kidney failure by rescuing defects of sulfides oxidation and ameliorating oxidative stress, despite only incomplete normalization of kidney CoQ levels and lack of rescue of CoQ-dependent respiratory enzymes activities. Liver and kidney lipidomics, and urine metabolomics analyses, did not show CoQ metabolites. To further demonstrate that sulfides metabolism defects cause oxidative stress in CoQ deficiency, we show that silencing of sulfide quinone oxido-reductase (SQOR) in wild-type HeLa cells leads to similar increases of reactive oxygen species (ROS) observed in HeLa cells depleted of the CoQ biosynthesis regulatory protein COQ8A. While $CoQ_{10}$ supplementation of COQ8A depleted cells decreases ROS and increases SQOR protein levels, knock-down of SQOR prevents $CoQ_{10}$ antioxidant effects. We conclude that kidney failure in CoQ deficiency-associated NS is caused by oxidative stress mediated by impaired sulfides oxidation and propose that CoQ supplementation does not significantly increase the kidney pool of CoQ bound to the respiratory supercomplexes, but rather enhances the free pool of CoQ, which stabilizes SQOR protein levels rescuing oxidative stress.

## 1. Introduction

Deficiency of coenzyme $Q_{10}$ ($CoQ_{10}$, the major form of CoQ in humans) is one of the few readily treatable mitochondrial diseases. However, the response to supplementation varies among phenotypes. Nephrotic syndrome (NS), either in isolation or as part of an infantile multi-systemic disease, is the most common manifestation associated with $CoQ_{10}$ deficiency due to mutations in genes encoding for proteins involved in $CoQ_{10}$ biosynthesis (primary $CoQ_{10}$ deficiency), and is the most responsive to $CoQ_{10}$ supplementation [1]. However, the mechanism of the selective responsiveness of NS is not clear, because, paradoxically, kidney is one of the organs with the poorest uptake of exogenous $CoQ_{10}$ [2,3].

$CoQ_{10}$ is a lipid molecule present in all cell membranes, where exerts a variety of biological functions [4]. For example, it carries electrons in the mitochondrial respiratory chain, acts as antioxidant, and, due to his redox properties, functions as cofactor for numerous enzymes, as sulfide quinone oxido-reductase (SQOR), the first enzyme of the sulfide ($H_2S$) oxidation pathway, or electron-transferring flavoprotein dehydrogenase (ETFDH), involved in fatty acid oxidation [5]. Whether there is a pool of CoQ responsible for all its biological functions, or whether two pools, one bound to mitochondrial respiratory supercomplexes, and one free, have separate functions, is still under debate [6]. Considering the multiple functions of $CoQ_{10}$, the clinical heterogeneity and variable response to supplementation of $CoQ_{10}$ deficiency is not surprising and may reflect tissue-specific pathomechanistic effects.

To address these issues, we have been studying *Pdss2*$^{kd/kd}$ mice, which carry a spontaneous mutation in the subunit 2 of polyprenyl-diphosphate synthase, the first enzyme involved in the CoQ

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biosynthetic pathway [7]. In humans, molecular defects in *PDSS2* manifest with nephrotic syndrome and encephalopathy [8], whereas *Pdss2*$^{kd/kd}$ mice develop isolated nephropathy with proteinuria that progresses to lethal renal failure as the only clinical manifestation of the disease [9], thus reproducing the human disease caused by molecular defects in *COQ2*, *COQ6*, and *COQ8B* [10–15]. The predominant kidney phenotype in these mice has been attributed to renal CoQ deficiency producing respiratory chain deficiency, increased oxidative stress, or both [16,17]. We previously showed that mitochondrial loss triggered by oxidative stress causes kidney failure in *Pdss2*$^{kd/kd}$ mice. Despite the widespread deficiency of $CoQ_9$ (the predominant form of CoQ in mice) and mitochondrial respiratory chain deficiencies, only affected organs show oxidative stress, with increased reactive oxigen species (ROS) as first abnormality, occurring in pre-symptomatic stage of the disease [16]. Recently, we also observed that in 6 month-old *Pdss2*$^{kd/kd}$ mice (late stage of disease), kidney showed: (i) reduced protein levels of SQOR and downstream enzymes, (ii) accumulation of hydrogen sulfides, and (iii) depletion of glutathione (GSH). In *Pdss2*$^{kd/kd}$ mice, we also observed low levels of plasma and urine thiosulfate and increased blood C4-C6 acylcarnitines [18], indicative of inhibition of short-chain acyl-CoA dehydrogenase (SCAD), a known toxic effect of $H_2S$ [16].

ROS production has been implicated as mechanism of $H_2S$ toxicity, for example by inhibiting cytochrome *c* oxidase (COX, complex IV) or by modulating the levels of GSH [19–21], leading us to hypothesize that in CoQ deficiency-associated NS, oxidative stress is caused by $H_2S$ oxidation impairment. Therefore, to investigate the mechanisms underlying $CoQ_{10}$ deficiency-related NS, and understand $CoQ_{10}$ mechanisms of action, we assessed the effects of long-term $CoQ_{10}$ oral supplementation on clinical, biochemical and molecular abnormalities in kidney of *Pdss2*$^{kd/kd}$ mice. Since the negligible effects of $CoQ_{10}$ supplementation can be explained by the strong lipophilicity of $CoQ_{10}$, which reaches mitochondria in small proportion, we compared the effects of $CoQ_{10}$ supplementation with the effects of supplementation with idebenone (IDB), a synthetic molecule with quinone proprieties similar to $CoQ_{10}$, but with a shorter hydrocarbon chain responsible for its different role in the mitochondrial respiratory chain [22]. Hydrophilic ubiquinone analogs as IDB are less efficient than hydrophobic ubiquinone analogs in the generation of energy by the mitochondrial respiratory chain, since the specific actions of both kind of ubiquinones depends of their interaction with two different bind sites, and hydrophilic quinones may enhance oxidative stress by interaction with the electron escape sites on Complex I [23–25]. Although the results of our previous *in vitro* experiments suggested that primary $CoQ_{10}$ deficiencies should be treated with $CoQ_{10}$ supplementation but not with short-tail ubiquinone analogs, we observed that oxidative stress and cell death could be counteracted by administration of lipophilic or hydrophilic antioxidants [26].

## 2. Material and methods

### 2.1. Animals

#### 2.1.1. Care

B6/*Pdss2*$^{kd/kd}$ mice were purchased from Jackson laboratory. *Pdss2*$^{kd/kd}$ mice harbor a spontaneous mutation in the gene encoding the subunit 2 of polyprenyl-diphosphate synthase (*Pdss2*) and have been previously described. All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Columbia University Medical Center, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed and bred according to international standard conditions, with a 12 h light, 12 h dark cycle and free access to food and water.

Mutant and controls animals were euthanized at 6 months (late-stage of the disease) and at 20 months ($CoQ_{10}$ treated animals, the only group that survived) unless differently specified. Affected (kidney) and two unaffected tissues (brain and liver) were quickly removed and frozen in the liquid phase of isopentane, pre-cooled toward its freezing point (−80 °C) with dry ice, or fixed in a solution of 4% paraformaldehyde. Urine were collected at 6 months of age. Blood was extracted from the heart and collected in tubes with EDTA. Plasma was obtained from blood by centrifugation at 4 °C at 2500 rpm for 15 min and kept at −80 °C.

#### 2.1.2. Groups

Regular chow was administered to wild-type and mutant mice until 3 months of age (age of phenotypic onset), followed by either regular, 0.5% Idebenone (IDB) or 0.5% Coenzyme $Q_{10}$ (CoQ) supplemented food. $CoQ_{10}$ and Idebenone supplemented food was purchased from Envigo (Envigo, Somerset, NJ). All the experiments were performed in 5–10 animals for group unless differently specified.

### 2.2. Assessment of kidney function

Proteins and creatinine levels in mice urine were measured commercially by Antech® Diagnostic (Fountain Valley, CA). Protein concentration was also assessed using a commercial kit (Chemistrip® 10 with SG, Roche Diagnostic, Germany) following the manufacturer's instructions.

### 2.3. $CoQ_9$ and $CoQ_{10}$ measurement

$CoQ_9$ (the main CoQ specie in mice) and $CoQ_{10}$ were extracted from kidney, brain and liver, in 1-propanol. The lipid component of the extract was separated by high-performance liquid chromatography (HPLC) on a reverse-phase Waters Symmetry C18 3.5 µm, 4.6 × 150 mm (Waters Corp., Milford, MA, USA), using a mobile phase consisting of methanol, ethanol, 2-propanol, acetic acid (500:470:15:15), and 50 mM sodium acetate at a flow rate of 0.8 ml/min. The electrochemical detector, ESA Coulochem II (ESA Inc., Chelmsford, MA, USA), was used with the following settings: guard cell (upstream of the injector) at 650 mV, conditioning cell at −650 mV (downstream of the column), followed by the analytical cell at +450 mV. CoQ concentration was estimated by comparison of the peak area with those of standard solutions of known concentration and expressed in micrograms per gram of protein [8].

### 2.4. Mitochondrial respiratory chain enzymes activities

CoQ-dependent respiratory chain activities (NADH cytochrome *c* reductase, complex I + III, and Succinate cytochrome *c* reductase, complex II + III), and citrate synthase (CS) activities were measured spectrophotometrically [16]. All following reactions were measured at 30 °C. Complex I + III activity was measured in the presence of 10 mM potassium cyanide, 2 mM NADH, and 1 mM cytochrome *c*, as the rotenone-sensitive reduction of cytochrome *c* assessed at 550 nm. The results were expressed in nmol reduced cyt c/min/mg protein. Complex II + III activity was measured in the presence of 10 mM KCN and 30mMsuccinate in $KH_2PO_4$ buffer (pH 7.5). The reaction was initiated by addition of 1 mM cytochrome *c* and the decrease in absorbance was monitored at 550 nm. The results were expressed in nmol reduced cyt c/min/mg protein. CS activity was measured following the reduction of 1 mM 5,50-dithiobis (2-nitrobenzoic acid) in 1 M Tris–HCl (pH 8.1) at 412 nm for 2 min in the presence of 10 mM acetyl-CoA, and 10 mM oxaloacetic acid, and expressed as nmol/min/mg protein.

### 2.5. Respiratory super-complexes native gel

For separation and quantification of respiratory super-complexes, mitochondrial inner membranes were prepared according to published protocols [28]. Five milligram of kidney tissue were homogenized in 500 ml sucrose buffer (250 mM sucrose, 20 mM sodium phosphate,

pH 7.0) and solubilized by adding digitonin (2% w/v). Solubilized mitochondrial complexes were divided in two aliquots and separated using high resolution clear native buffer 3 [29]. To visualize the supercomplexes, gels were incubated at room temperature with specific complex I assay buffer (25 mg of NTB, 100 μl of NADH in 5 mM Tris/HCl, pH 7.4 buffer). The reaction was stopped after 5 min and scanned for densitometric quantitation. To assess complex III in-gel activity, gels were incubated in buffer containing 5 mg of diaminobenzidine dissolved in 10 ml of 50 mM sodium phosphate, pH 7.2, and acquired after 1 h incubation.

### 2.6. Morphology and tissue studies

All sections were examined using an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a computer assisted image analysis system, and images were captured with a QImaging Retiga EXi digital camera, using QCapture software (QImaging, Surrey, BC, Canada), and with a Zeiss AX10 epifluorescence microscope and processed by NIH ImageJ 1.50e software.

#### 2.6.1. Histology

To visualize histological features, as well as extent of renal damage, hematoxylin and eosin (H&E) staining was performed on kidney sections. Briefly, sections were deparaffinized and rehydrated using Trilogy™ (Cell Marque, Rocklin, CA), stained in filtered Harris hematoxylin (Hematoxylin Solution, Harris Modified, Sigma Aldrich, Saint Louis, MO) for 10 min, washed in 0.2% acetic acid for 30 s and rinsed in $dH_2O$. Sections were then stained in eosin (Eosin Y solution, alcoholic, Sigma Aldrich, Saint Louis, MO) for 1 min, dehydrated by passages in alcohol from 50% to 100% 1 min each and cleaned in xylene twice (2 min each). Coverslips were added using Permount (Permount Mounting Medium, Fisher Scientific, Hampton, NH). Renal Damage Score (RDS) was determined as in [27]. The sections were examined blindly and scored as follows: 0 = no tubular dilatation and no cell infiltrates. 1 = small focal areas of cellular infiltration and tubular dilatation involving < 10% of the cortex; 2 = involvement of up to 25% of the cortex; 3 = involvement of up to 50% of the cortex; 4 = extensive damage involving > 75% of the cortex. The presence or absence of glomerulosclerosis was determined as in [9].

#### 2.6.2. Oxidative stress

Kidney were fixed in 10% neutral buffered formalin and embedded in paraffin using standard procedures. Sections (3 μm) were used for immunohistochemical studies to assess oxidative stress. For deparaffinization, rehydration and unmasking of the antigen the buffer Trilogy™ (Cell Marque, Rocklin, CA) was used. Section were then washed three times with PBS, incubated for 1 h in blocking buffer (PBS, BSA 1%, Triton X 0.5%) and then incubated with the primary antibody overnight. After three washes in PBS, sections were incubated for 1 h at room temperature with the secondary antibody, washed again with PBS three times, incubated with Hoechst (Hoechst 33342, Thermo Fisher, Waltham, MA) for 10 min, washed once with PBS and finally covered using Vectashield (Vectashield mounting medium H-1000, Vector Laboratories, Burlingame, CA). Rabbit anti-4-hydroxynonenal antiserum (HNE11-S; AlphaDiagnostic International, Inc., 1:1000) was used to detect lipid peroxidation [16]; rabbit polyclonal anti-nitrotyrosine (PNK) (sc-55256, Santa Cruz Biotechnology Inc., Santa Cruz, CA, 1:1000) was used to detect protein oxidation [30]. As secondary antibodies, we used species specific, Alexa Fluor™ 488 antibody (Sigma Aldrich, Saint Louis, MO, 1:2000).

Images where taken with a Zeiss AX10 epifluorescence microscope and processed with Image-J to quantify fluorescence intensity. Single glomeruli where analyzed from ten fields for each kidney. The fluorescence intensity of single glomeruli was measured and background signal was subtracted.

### 2.7. Assessment of sulfides oxidation pathway

To assess the $H_2S$ oxidation pathway, we measured mRNA and protein levels of $H_2S$ oxidation enzymes (SQOR, TST, ETHE1, and SUOX). To measure mRNA, quantitative RT–PCR was performed using TaqMan® Assays with the following Applied Biosystems probes: *SQRDL*, Mm00502443_m1; *TST*, Mm01195231_m1; *SUOX*, Mm00620388_g1; *ETHE1*, Mm00480916_m1. Expression of the target genes was calculated by $-\Delta\Delta CT$ method and normalized to the expression of *GAPDH* (#4308313). The experiments were performed in technical triplicates of at least three biological replicates as described [18].

To measure protein levels, we performed western blots. Proteins were extracted from cell pellets by sonication and from mouse tissues by mechanical homogenization in lysis buffer (50 nM Tris HCl, 150 mM NaCl, 1 mM EDTA). To prevent protein degradation a protease inhibitor cocktail (Complete Mini®, EDTA-free, 11836170001, Roche) was added to the protein extract and the samples were kept at −80 °C. Cell lysates were quantitated for total protein content using the Bradford system (ThermoFisher, Waltham, MA); 10–20 μg of proteins were loaded and analyzed by electrophoresis in a 12–15% PAGE gel or Novex 10–20% Glycine Gel (EC61355, Invitrogen, Carlsbad, CA). After electrophoresis, proteins were transferred to a PVC membrane (IPFL00010, Immobilon-FL). Membranes where blocked in PBT with 2.5–5% milk before incubation with the following antibodies: rabbit anti-SQOR (1:1000, ab118772 Abcam, Cambridge, UK); rabbit anti-TST, (1:1000, ab155320, Abcam); mouse anti-SUOX (1:1000, ab57852, Abcam, Cambridge, UK); rabbit anti-ETHE1 (1:1000, ab154041, Abcam, Cambridge, UK); rabbit anti-TOM20 (1:500, sc-11,415, Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-Vinculin (1:5000, ab18058, Abcam, Cambridge, UK); secondary rabbit and mouse HRP-conjugated antibodies (1:2000, A9044 and A0545, Sigma Aldrich, Saint Louis, MO). Protein bands were visualized by chemiluminescence, using ECL reagents (GE Healthcare, Little Chalfont, UK). Intensity of the bands was quantified with ImageJ (NIH), according to [18].

### 2.8. GSH measurement

The level of total GSH (T-GSH) was measured as described in [31]. Briefly, frozen tissue were homogenized and centrifuged for 20 min at 12000 rpm. Proteins were quantified by Bradford assay and 25 μg of protein were diluted in 100 μl of 6% Ortophosphoric acid. The supernatant was then diluted 1:20 in assay buffer (0.1 M potassium phosphate buffer pH 7.4; 5 mM EDTA; 0.66 mM DTNB; 0.5 μM NADPH). A standard calibration curve was prepared ranging from 0 to 12 mg/ml of GSH. In a 1 ml cuvette, 5 μl of sample/standard, 400 μl of assay buffer and 5 μl (3 Units) of Glutathione Reductase (5 μg/μl) were mixed. After 3 min of incubation, the absorbance was measured at A412nm for 3 min. Total GSH concentration was then extrapolated using the standard curve and expressed as expressed pmol/mg protein.

### 2.9. Ultra-carrying out liquid chromatography-mass spectrometer (MS/MS), analysis of CoQ excretion metabolites

Lipids extracts were obtained by mixing urine with 1-propanol or by hexane extraction [32,33]. Samples were analyzed using an Acquity ultra carrying out liquid chromatography system H-Class (WatersCorporation) coupled to a Xevo TQS detector of mass spectrometer (MS/MS) with an electrospray ionization (Waters Corporation). The analytical separation column was a BEH C18, 1.7 μm, 2.1 × 50 mm column (Waters, Spain) [32]. The mobile phase consisted of methanol and 0.1% Formic acid at the constant flow rate of 0.45 ml/min. Source and desolvation temperatures were set at 140 and 500 °C, respectively. Nitrogen was used as both cone gas (150 l/h) and desolvation gas (800 l/h), and argon was used as collision gas (0.14 ml/min). Mass spectrometry analyses were carried out in full scan mode between 300 and 600 uma, as well as 600 and 950 uma to improve the sensitivity of

the analysis.

### 2.10. Serum acylcarnitines determination

#### 2.10.1. Samples preparation

To investigate fatty acid oxidation, we performed serum acylcarnitines profile. All solvents for sample preparation and UPLC/MS/MS analysis were LC/MS grade (Fisher Scientific; Pittsburgh, PA). Free carnitine and fatty acylcarnitine standards were purchased from Toronto Research Chemicals (Toronto, Ontario) and R&D Systems, Inc. (Minneapolis, MN). Deuterated internal standards were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). All the procedures were carried out on ice or at 4 C. An aliquot of 100 µl methanol containing internal standards was added to 50 µl of plasma in a 1.5 ml microcentrifuge tube. After vortexing, 400 µl acetonitrile were added to the sample. Samples were vortexed at 2000 rpm for 30 min and centrifuged at 20,000*g* for 10 min. The clear supernatant was transferred to an Agilent micro sampling vial (Agilent Technologies, Cat No 5184-3550, Santa Clara, CA). The supernatant was evaporated to dryness with nitrogen at room temperature and subsequently reconstituted in 100 µl of 1:9 (v:v) methanol/water containing 10 mM ammonium acetate and 10 mM heptafluorobutyric acid for injection.

#### 2.10.2. LC-MS conditions

All experiments were carried out on an Agilent 6410 Triple Quad LC-MS/MS system. 2.5 µl were loaded onto an Agilent Poroshell 120 EC-C18 column (3.0 mm inner diameter × 50 mm with 2.7 µm particles). The column was maintained at 50 °C throughout analysis. The initial conditions were as follows: 500 µl/min with a mobile phase composition of 98% solvent A (water containing 10 mM ammonium acetate and 10 mM heptafluorobutyric acid) and 2% solvent B (methanol containing 10 mM ammonium acetate and 10 mM heptafluorobutyric acid). Initial conditions were maintained for 1 min. Solvent B was lineally increased to 90% over 5 min and maintained for 1 min. The column was cleaned with 98% solvent B for 3 min and conditioned with 2% solvent B for 3 min before the next injection. Carnitine and acylcarnitines of interest elute between 1.3 and 6.8 min. Positive electrospray tandem mass spectrometry (ESI-MS/MS) under multiple reactions monitoring mode (MRM) was performed employing the following parameters: capillary voltage, 2000 V; gas temperature, 350 °C; gas flow, 13 l/min; nebulizer, 60 psi. Different species were identified by comparing the retention times of experimental compounds with those of authentic standards. Concentrations of carnitine and acylcarnitines in the serum were quantified by comparing integrated peak areas for those of each carnitines against those of known amounts of purified standards.

### 2.11. Kidney and liver lipidomics

Analysis of lipids in liver and kidney was performed using High Performance Liquid Chromatography-Mass Spectrometry. Lipid extracts were prepared using a modified Bligh and Dyer procedure as described previously [34,35], spiked with appropriate internal standards, and analyzed using a 6490 Triple Quadrupole LC/MS system (Agilent Technologies, Santa Clara, CA). Glycerophospholipids and sphingolipids were separated with normal-phase HPLC as described before [35], with few modifications. An Agilent Zorbax Rx-Sil column (inner diameter 2.1 × 100 mm) was used under the following conditions: mobile phase A (chloroform:methanol:1 M ammonium hydroxide, 89.9:10:0.1, v/v) and mobile phase B (chloroform:methanol:water:ammonium hydroxide, 55:39.9:5:0.1, v/v); 95% A for 2 min, linear gradient to 30% A over 18 min and held for 3 min, and linear gradient to 95% A over 2 min and held for 6 min. Sterols and glycerolipids were separated with reverse-phase HPLC using an isocratic mobile phase as before [35] except with an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 100 mm). Quantification of lipid species was accomplished using multiple reaction monitoring (MRM) transitions developed in earlier studies [35] in conjunction with referencing of appropriate internal standards: PA 14:0/14:0, PC 14:0/14:0, PE 14:0/14:0, PI 12:0/13:0, PS 14:0/14:0, SM d18:1/12:0, D7-cholesterol, CE 17:0, MG 17:0, 4ME 16:0 diether DG, D5-TG 16:0/18:0/16:0 (Avanti Polar Lipids, Alabaster, AL).

### 2.12. In vitro studies

#### 2.12.1. Cell culture and $CoQ_{10}$ supplementation

To generate Hela cells depleted of COQ8A, cells were stable transfected with scramble shRNA-pLKO plasmid (used as control EV) or COQ8A-specific TRC shRNA-pLKO plasmid construct (SHCLNG-NM_020247 MISSION® shRNA Bacterial Glycerol Stock; Sigma Aldrich).

EV and COQ8A shRNA cells were plated at concentration of $0.5 \times 10^6$/ml in six well plates and cultured in DMEM. After 24 h, the medium was supplemented with 10% FBS ± 10 µM of $CoQ_{10}$ (Hydro Q Sorb Powder, Tishcon Corp., USA) for 72 h. Transient knockdown of SQOR in wild-type and COQ8A depleted HeLa cells was obtained incubating cells for 6 h in OptiMEM medium supplemented with 5 ml MEM vitamins and 5 ml MEM non-essential amino acids, 25 pmol of SQRDL Silencer Select Validated siRNA (Ambion 4390824), and Lipofectamine 2000.

After 6 h, 10% FBS ± 10uM of $CoQ_{10}$ was supplemented and cells where incubated for other 42 h.

#### 2.12.2. Determination of superoxide production

To estimate production of ROS, cells were washed in PBS and treated with 5 mM of MitoSOX (Molecular Probes - M36008) for 30 min. at 37 °C, nuclei were stained with 0.1µg/ml of Hoechst 33342 (Thermo Scientific – 62249). Cells were washed twice in PBS and images where captured with fluorescence microscope (Nikon Eclipse TE-2000). Images were elaborated and fluorescence intensity was measured with ImageJ.

#### 2.12.3. Determination of protein steady-state levels by immunoblotting

Steady-state protein levels of COQ8A and SQOR were measured by western blot analysis. Proteins were extracted from cell pellets by sonication in water. To prevent protein degradation, a protease inhibitor cocktail (Complete Mini®, EDTA-free, 11836170001, Roche) was added to the protein extract and the samples were kept at −80 °C. Cell lysates were quantitated for total protein content using the BCA system (ThermoScience) and analyzed by electrophoresis in Novex 10–20% Glycine Gel (EC61355, Invitrogen) loading 20 µg of protein for sample. After electrophoresis, proteins were transferred to a PVC transfer membrane (IPFL00010, Immobilon-FL). Membranes were blocked in PBT with 5% milk before incubation with the following antibodies: rabbit anti-SQRDL (1:1000, 17256-1-AP Proteintech); rabbit anti-ADCK3/CABC1 (1:1000, Thermo Scientific, Pierce, PA5-13906) mouse anti-vinculin (1:5000, Abcam ab18058); secondary rabbit and mouse HRP (1:2000, Sigma A9044 and A0545). Protein bands were visualized by chemiluminescence, using ECL reagents (GE Healthcare). Intensity of the bands was quantified with ImageJ (NIH).

### 2.13. Statistical analysis

For the survival curve, the longrank (or Mantel-Cox) test was used; in order to compare results of CoQ, RCA, Western blots, acylcarnitine, GSH, lipidomics and qPCR measurement, the Mann–Whitney non-parametric *U* test was used, unless specified otherwise. To compare urine proteinuria unpaired *t*-test was used. For the quantification of fluorescence intensity in the *in vitro* and *in vivo* experiments one way ANOVA test was used. For statistical analysis, GraphPad Prism v5 was used. Data are expressed as mean ± SD of at least three experiments for group. A value of $p < 0.05$ was considered to be statistically significant. * indicates a value of $p < 0.05$, ** indicates a value of $p < 0.01$, and *** indicates a value of $p < 0.001$.

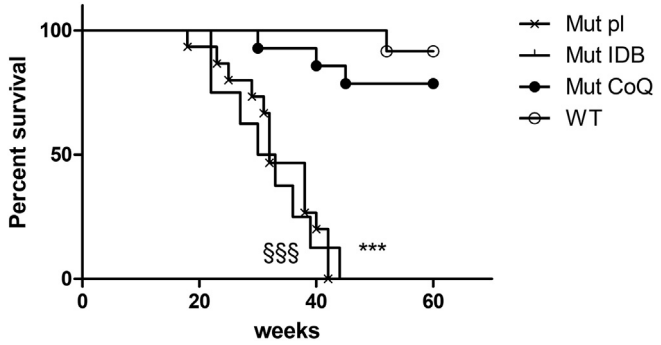


**Fig. 1.** Survival curve in *Pdss2*$^{kd/kd}$ mice: $CoQ_{10}$ supplementation significantly increases survival of mutant mice. Mut Placebo *vs* WT: $p < 0.001$, §§§; Mut IDB *vs* WT: $p < 0.001$, *** (Mantel-Cox test). Mut placebo = 15, Mut IDB = 8, Mut CoQ = 9, WT = 15. Mut = mutant; IDB = Idebenone; CoQ = $CoQ_{10}$; WT = wild type.

## 3. Results

### 3.1. $CoQ_{10}$ supplementation increases the life span of *Pdss2*$^{kd/kd}$ mutant animals and stabilizes proteinuria

To evaluate the effect of our treatments on the life span of *Pdss2*$^{kd/kd}$, we compared survival curve of placebo, $CoQ_{10}$, and IDB treated animals. While survival of mutant animals was dramatically prolonged by $CoQ_{10}$ supplementation, it was not improved by IDB treatment (Fig. 1).

Protein/creatinine ratio was significantly increased in 6 month-old mutant animals in placebo compared with wild-type animals, and was reduced by $CoQ_{10}$ supplementation (Table 1).

Mutant animals treated with placebo showed proteinuria starting at 3 months of age. Proteinuria worsened in 6 month-old placebo and IDB treated mutant animals, but not in $CoQ_{10}$-treated *Pdss2*$^{kd/kd}$ mice at ages 3, 6, 9 and 12 month-old (Suppl. Table 1), indicating that $CoQ_{10}$ supplementation prevents the progression of the disease to renal failure.

### 3.2. $CoQ_{10}$ supplementation preserves kidney morphology

Kidney of 4 month-old (age of onset of disease) *Pdss2*$^{kd/kd}$ mice showed tubular interstitial nephritis and collapsing glomerulopathy, absent in younger mutant mice [16,36]. To assess the effects of the treatments on kidney morphology, we performed H&E staining, and quantified kidney damage. Kidney of 6 and 20 month-old mutant animals supplemented with $CoQ_{10}$ showed preserved histological structure. In contrast, IDB supplementation had no beneficial effects on the tissue morphology, as glomeruli were disrupted (Fig. 2). Renal Damage Score (RDS) was 3+ in mutant animals in placebo and IDB; 0 in wild-type animals, and 1 in mutant animals treated with $CoQ_{10}$. We found glomerulosclerosis in mutant animals in placebo and IDB. On the contrary, despite minor cortical damage, we did not detect any sign of glomerulosclerosis after $CoQ_{10}$ treatment in mutant mice (RDS = 1) (Fig. 2).

These data, together with the results of survival and proteinuria, indicate that $CoQ_{10}$ supplementation prevents kidney failure in *Pdss2*$^{kd/kd}$ mice.

### 3.3. $CoQ_{10}$ supplementation partially increases CoQ levels in kidney of *Pdss2*$^{kd/kd}$ mutant mice

To assess $CoQ_{10}$ organs uptake, $CoQ_9$ and $CoQ_{10}$ levels were measured in affected tissue (kidney), two unaffected tissues used as control (brain and liver), and plasma. Consistently with our previously published data (16), kidney of 6 month-old mutant animals in placebo showed significantly decreased $CoQ_9$ and $CoQ_{10}$ levels compared with wild-type animals (kidney $CoQ_9$: Mut placebo 40% of WT, $p = 0.0012$; kidney $CoQ_{10}$: Mut placebo 17% of WT, $p = 0.0003$; Table 2, Fig. 3A, B). Also brain and liver, despite being clinically unaffected, showed significantly decreased $CoQ_9$ and $CoQ_{10}$ levels (Suppl. Table 2, Suppl. Fig. 1A, B, D, E).

The levels of $CoQ_9$ and $CoQ_{10}$ in kidney of 6 month-old animals supplemented with $CoQ_{10}$, were increased compared to animals in placebo (kidney $CoQ_9$: WT CoQ 310% of placebo, $p = 0.0025$; kidney $CoQ_{10}$: WT CoQ 146% of placebo, $p = 0.045$; kidney $CoQ_9$: Mut CoQ 145% of placebo, $p = 0.32$; kidney $CoQ_{10}$: Mut CoQ 160% of placebo, $p = 0.073$; Table 2, Fig. 3A, B). However, they were still significantly reduced in mutant compared with wild-type animals (kidney $CoQ_9$: Mut CoQ 19% of WT, $p = 0.016$; kidney $CoQ_{10}$: Mut CoQ 19% of WT, $p = 0.016$; Table 2, Fig. 3A, B).

On the contrary, levels of $CoQ_9$ and $CoQ_{10}$ were not increased in kidney of 20 month-old mutant animals supplemented with $CoQ_{10}$ (kidney $CoQ_9$: Mut CoQ 34% of WT, $p = 0.0012$; kidney $CoQ_{10}$: Mut CoQ 23% of WT, $p = 0.0017$; Table 2, Fig. 3A, B).

To assess whether levels of $CoQ_{10}$ in kidney of 20 month-old animals supplemented were low because exogenous $CoQ_{10}$ was metabolized, we measured $CoQ_9$ and $CoQ_{10}$ levels in plasma of 20 month-old CoQ-treated animals. However, the levels of $CoQ_{10}$ were comparable in plasma of treated mutant and wild-type animals (Suppl. Table 2, Suppl. Fig. 1C, F).

To assess whether exogenous $CoQ_{10}$ accumulates in the liver, as previously reported [37,38], we measured $CoQ_9$ and $CoQ_{10}$ in liver of 6 month-old and 20 month-old animals and found that $CoQ_{10}$ supplementation increased liver $CoQ_{10}$ levels in mutant and wild-type animals proportionally to the duration of the supplementation, and $CoQ_9$ and $CoQ_{10}$ in liver of treated mutants were comparable with treated wild-type animals (Suppl. Table 2, Suppl. Fig. 1B, E).

As expected, due to the poor bioavailability of $CoQ_{10}$, supplementation did not change levels of $CoQ_9$ and $CoQ_{10}$ in brain of 6 and 20 month-old treated animals (Suppl. Table 2, Suppl. Fig. 1A, D).

IDB supplementation did not affect $CoQ_9$ and $CoQ_{10}$ values compared to placebo animals (Table 2, Fig. 3A, B, and Suppl. Table 2, Suppl. Fig. 1A, B, D, E).

These results indicate that $CoQ_{10}$ supplementation causes accumulation of CoQ in plasma and liver, and partially and transiently increases CoQ levels in kidney.

**Table 1**
Protein and creatinine levels in urine of 6 mo *Pdss2*$^{kd/kd}$ mice.

| | WT pl 6 mo | Mut pl 6 mo | WT CoQ 6 mo | Mut CoQ 6 mo |
|---|---|---|---|---|
| Protein-urine (mg/dL) | 275.2 ± 15.8 | 612.1 ± 183.3 | 223.6 ± 210.4 | 388.8 ± 334.3 |
| Creatinine (mg/dL) | 65.1 ± 21.1 | **30.6 ± 3.8*** | 47.1 ± 11.6 | **32.2 ± 2.1*** |
| Urine protein/creatinine | 2.9 ± 0.6 | **20.0 ± 5.5*** | 4.3 ± 3.0 | 10.8 ± 8.6 |

WT = wild type; Mut = mutant, mo = month-old; pl = placebo; CoQ = $CoQ_{10}$; N = 3 for group. Bold characters = statistical significance.
* $p < 0.05$ (unpaired *t*-test).

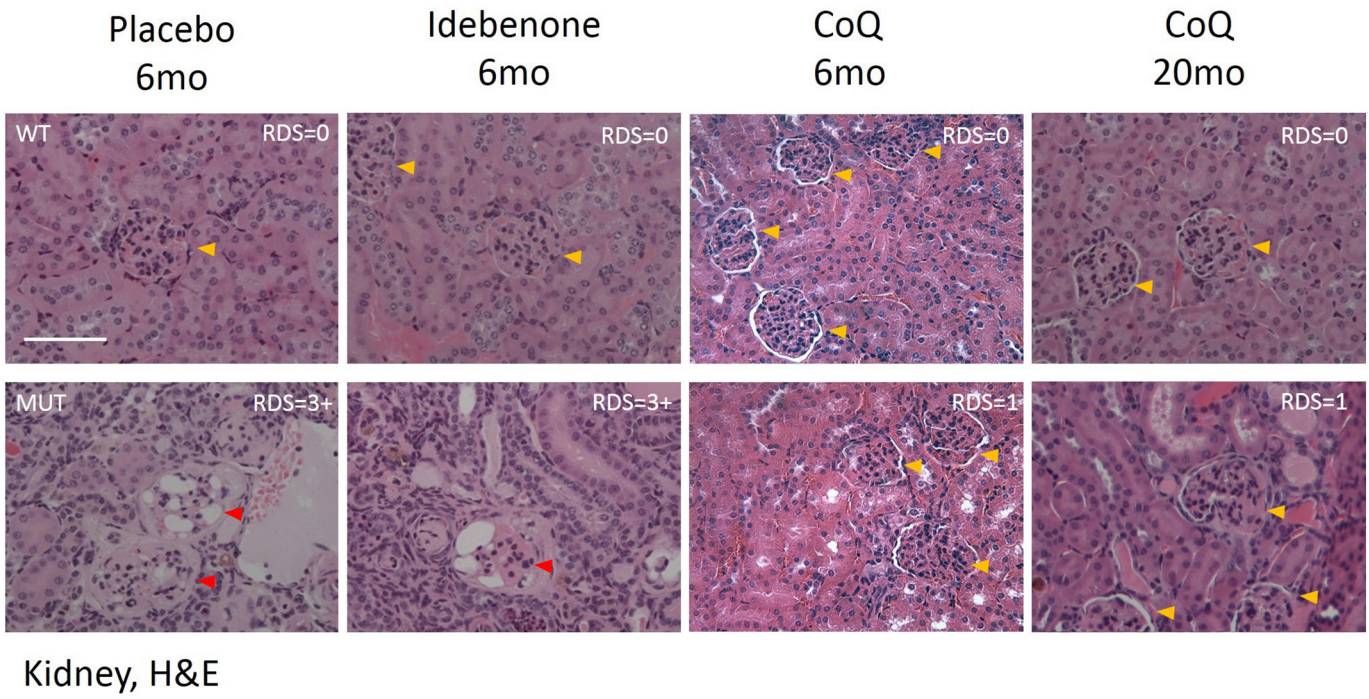


**Fig. 2.** Kidney morphology: $CoQ_{10}$ supplementation preserves kidney structure in *Pdss2*$^{kd/kd}$ mutant mice. Representative images of H&E staining in kidney of wild-type (WT) and mutant (MUT) animals (N = 3 for group). RDS = Renal Damage Score. Yellow arrows indicate healthy glomeruli, red arrows indicate disrupted glomeruli. Magnification: 20×; CoQ = $CoQ_{10}$; 6 mo = 6 month old; 20 mo = 20 month old, H&E = Hematoxylin - Eosin; scale bar = 100 μm.

**Table 2**
CoQ levels in kidney of *Pdss2*$^{kd/kd}$ mice.

| Kidney | WT pl 6 mo | Mut pl 6 mo | WT IDB 6 mo | Mut IDB 6 mo | WT CoQ 6 mo | Mut CoQ 6 mo | WT CoQ 20 mo | Mut CoQ 20 mo |
|---|---|---|---|---|---|---|---|---|
| $CoQ_9$ | 1867 ± 1122 | **756.8 ± 336.2**** | 3812 ± 2030 | **596 ± 291.1***** | 5785 ± 1391 | **1098 ± 606.4*** | 1312 ± 573 | **450.6 ± 187.4**** |
| $CoQ_{10}$ | 493.1 ± 178.4 | **83.6 ± 26.2***** | 591.8 ± 212.6 | **67.4 ± 38.5***** | 721.8 ± 183.3 | **133.7 ± 47.5*** | 388.4 ± 210.1 | **91.28 ± 27**** |

CoQ values are expressed as mean ± standard deviation μg/g protein. WT = wild type; Mut = mutant, mo = month-old; pl = placebo; IDB idebenone; CoQ = $CoQ_{10}$; N = 7 per group. Bold characters = statistical significance.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ (Mann Whitney test).

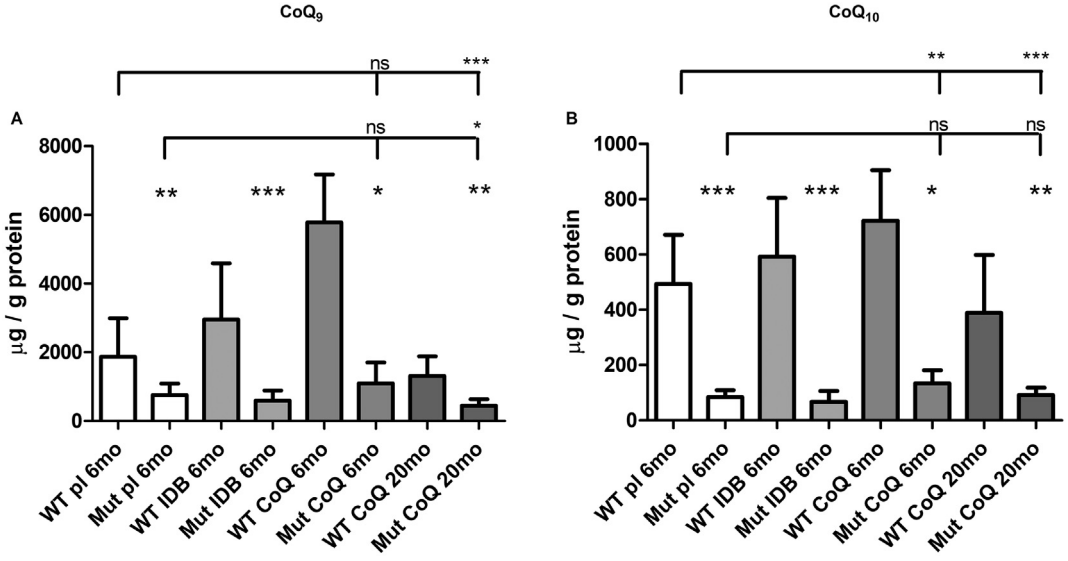


**Fig. 3.** CoQ levels in kidney of *Pdss2*$^{kd/kd}$ mice: $CoQ_{10}$ supplementation partially increases CoQ levels in 6 month-old mutant and wild-type mice. Amount of $CoQ_9$ (A) and $CoQ_{10}$ (B). Data are represented as mean ± standard deviation (N = 7). Mutant mice (Mut) are compared to age-matched controls (WT) under same treatment (pl = placebo; CoQ = $CoQ_{10}$; IDB = Idebenone); 6 mo = 6 month old; 20 mo = 20 month old. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant (Mann Whitney test).

**Table 3**
CoQ-dependent respiratory chain enzymes activities in kidney of *Pdss2*$^{kd/kd}$ mice.

| Kidney | WT pl 6 mo | Mut pl 6 mo | WT IDB 6 mo | Mut IDB 6 mo | WT CoQ 6 mo | Mut CoQ 6 mo | WT CoQ 20 mo | Mut CoQ 20 mo |
|---|---|---|---|---|---|---|---|---|
| I + III | 0.21 ± 0.1 | 0.16 ± 0.09 | 0.13 ± 0.08 | 0.08 ± 0.04 | 0.3 ± 0.05 | 0.57 ± 0.24 | 0.15 ± 0.11 | **0.05 ± 0.03**** |
| II + III | 0.18 ± 0.03 | 0.11 ± **0.06*** | 0.18 ± 0.1 | 0.09 ± 0.04 | 0.18 ± 0.07 | 0.11 ± 0.05 | 0.15 ± 0.03 | **0.08 ± 0.04**** |
| CS | 3.18 ± 1.76 | 3.24 ± 0.98 | 2.63 ± 1.87 | 2.2 ± 1.09 | 7.34 ± 2.43 | 6.68 ± 2.94 | 2.62 ± 1.34 | 3.4 ± 1.77 |

Enzyme activities values are expressed as mean ± standard deviation micromoles per minute per mg of protein. WT = wild type; Mut = mutant, mo = month-old; pl = placebo; IDB idebenone; CoQ = $CoQ_{10}$; CS = citrate synthase; I + II = complexes I + II; II + III = complexes II + III; N = 7 per group. Bold characters = statistical significance.

* $p < 0.05$.

** $p < 0.01$ (Mann Whitney test).

### 3.4. $CoQ_{10}$ supplementation does not rescues CoQ-dependent enzymes activities in kidney of *Pdss2*$^{kd/kd}$ mutant mice

To assess the effects of supplementations on mitochondrial bioenergetics, we measured the activities of the CoQ-dependent enzymes, complexes I + III and II + III.

In kidney of 6 month-old mutant animals in placebo complex I + III activity was slightly reduced compared with wild-type animals, and in 6 month-old mutant animals supplemented with $CoQ_{10}$, it was slightly increased, compared with wild-type animals, although these differences were not statistically significant (Table 3, Fig. 4A, B). On the contrary, complex II + III activity was significantly reduced in kidney of 6 month-old mutant animals in placebo, and showed a trend toward reduction in mutant animals in $CoQ_{10}$, compared with wild-type animals (Table 3, Fig. 4A, B). Importantly, both enzymes activities were significantly decreased in 20 month-old mutant animals supplemented with $CoQ_{10}$ compared to wild-type (activity levels Mut/WT ratio; kidney complex I + III: placebo 6 mo 0.75 ± 0.44; CoQ 6 mo 1.91 ± 0.81; CoQ 20 mo 0.37 ± 0.25, p = 0.0093; kidney complex II + III: placebo 6 mo 0.62 ± 0.33, p = 0.029; CoQ 6 mo 0.64 ± 0.30; CoQ 20 mo 0.50 ± 0.28, p = 0.0048; Table 3, Fig. 4A, B).

In accordance with the normal respiratory chain enzyme activities of complex I and III, we observed normal assembly levels of respiratory chain supercomplexes, S1 (I-III-IV) and S0 (I-III) in kidney of placebo 6 month-old animals (data not shown).

Complex I + III and II + III activities in brain and liver of mutant mice were comparable to wild-type before and after the supplementation (Suppl. Table 3, Suppl. Fig. 2A, B, D, E).

IDB supplementation did not affect complex II + III activity, but decreased complex I + III activity, compared to placebo animals (Table 3, Fig. 4A, B).

These results indicate that mitochondrial respiratory chain defects do not contribute to the pathogenesis of NS associated with CoQ deficiency.

### 3.5. Increased mitochondrial mass in kidney of *Pdss2*$^{kd/kd}$ mutant mice is a secondary effect of $CoQ_{10}$ supplementation

Loss of mitochondrial mass, due to abnormal mitophagy has been documented in CoQ deficiency *in vivo* and *in vitro* [39–41]. Therefore, we assessed kidney activity of citrate synthase (CS), a mitochondrial matrix enzyme, and the levels of TOM20, an outer mitochondrial membrane protein, both indices of mitochondrial mass. We found that CS activity was not altered in kidney of 6 month-old animals in placebo, IDB, and $CoQ_{10}$ supplementation, and showed a trend toward increase in $CoQ_{10}$ treated 20 month-old animals (Table 3, Fig. 4C). Since CS activity was increased in 20 month-old animals supplemented with $CoQ_{10}$, we normalized complexes activities over this index of mitochondrial mass, and we found them extremely reduced (activity levels Mut/WT ratio; kidney complex I + III/CS: placebo 6 mo 0.65 ± 0.42; IDB 6 mo 0.58 ± 0.35; CoQ 6 mo 1.67 ± 0.69; CoQ 20 mo 0.44 ± 0.26, p = 0.029; kidney complex II + III/CS: placebo 6 mo 0.89 ± 0.34; IDB 6 mo 0.41 ± 0.21, p = 0.036; CoQ 6 mo 0.91 ± 0.72; CoQ 20 mo 0.25 ± 0.08, p = 0.0003; Table 3).

In kidney of 6 month-old mutant animals on placebo, $CoQ_{10}$ or IDB, TOM20 levels were significantly decreased compared with wild-type animals (kidney TOM20 protein levels Mut/WT: placebo 6 mo 0.47 ± 0.28, p < 0.0001; IDB 6 mo 0.29 ± 0.19, p < 0.0001; CoQ 6 mo 0.44 ± 0.34, p = 0.0011; Fig. 5). In 20 month-old mutant animals supplemented with $CoQ_{10}$, the levels of TOM20 were comparable with controls (Fig. 5).

To assess the disease stage at which loss of mitochondria occurs, we measured TOM20 levels in kidney of 1 month-old mutant animals, and we found them comparable to wild-type (Fig. 5).

In brain and liver, CS activity was normal in all groups (Suppl. Table 3, Suppl. Fig. 2C, F). Interestingly, TOM20 levels were slightly

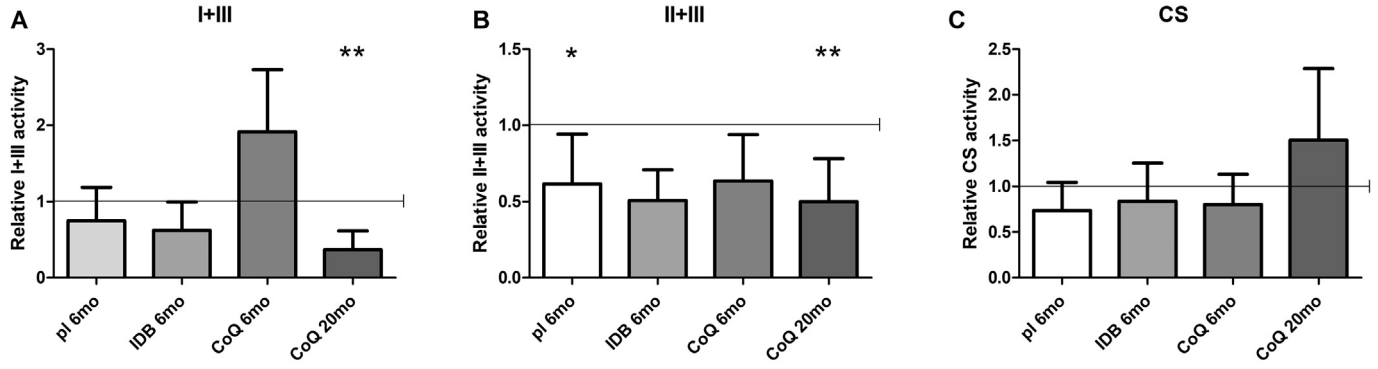


**Fig. 4.** CoQ-dependent respiratory chain enzymes activities in kidney of *Pdss2*$^{kd/kd}$ mice: $CoQ_{10}$ supplementation does not rescue mitochondrial respiratory chain function in mutant mice. Complexes I + III (A), II + III (B) and citrate synthase (C) measured by spectrophotometric assay in kidney homogenates. Values are represented as fold changes of mutant mice (Mut) compared to age-matched controls (WT) under same treatment (pl = placebo; CoQ = $CoQ_{10}$; IDB = Idebenone) (all set as 1, horizontal bar); CS = citrate synthase; I + II = complexes I + II; II + III = complexes II + III; 6 mo = 6 month old; 20 mo = 20 month old. Data are represented as mean ± standard deviation (N = 7 for group). * = p < 0.05, ** = p < 0.01 (Mann Whitney test).

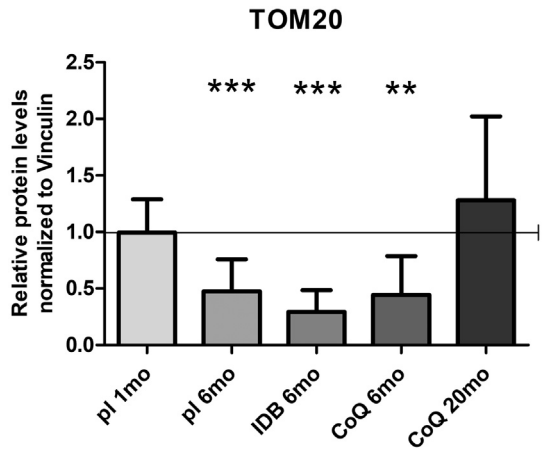


**Fig. 5.** Mitochondrial mass in kidney of *Pdss2*$^{kd/kd}$ mice: decrease of mitochondrial mass is a secondary effect of $CoQ_{10}$ deficiency. The levels of TOM20 were normalized to vinculin and represented as fold changes of mutant mice (Mut) compared to age-matched controls (WT) under same treatment (pl = placebo; CoQ = $CoQ_{10}$; IDB = Idebenone) (all set as 1, horizontal bar). 1 mo = 1 month-old, 6 mo = 6 month old; 20 mo = 20 month old. Data are represented as mean ± standard deviation (N = 4 for group). ** = $p < 0.01$, *** = $p < 0.001$ (Mann Whitney test).

reduced also in brain of 6 month-old untreated mutant animals compared with wild-type (Suppl. Fig. 3).

These results suggest that the decrease in mitochondrial mass is a secondary effect of $CoQ_{10}$ deficiency, and that the increase in mitochondrial mass is an effect of $CoQ_{10}$ supplementation secondary to the rescue of other biochemical and molecular abnormalities.

### 3.6. $CoQ_{10}$ supplementation reduces oxidative stress in kidney of *Pdss2*$^{kd/kd}$ mice

We previously showed that oxidative stress makers are increased only in kidney of *Pdss2*$^{kd/kd}$ mice [16]. To assess the efficacy of $CoQ_{10}$ and IDB as antioxidants, we performed immunofluorescence to measure levels of anti-nitrotyrosine and anti 4-hydroxynonenal, markers of protein and lipid oxidative stress, respectively. Surprisingly, $CoQ_{10}$ supplementation reduces oxidative stress damage in 6 and 20 month-old mutant animals, while IDB supplementation did not have any effect on oxidative stress markers (Fig. 6). These results support the role of oxidative stress in the pathogenesis of $CoQ_{10}$ deficiency and suggest that $CoQ_{10}$ and IDB have different antioxidant properties.

### 3.7. $CoQ_{10}$ supplementation rescues $H_2S$ oxidation pathway in kidney of *Pdss2*$^{kd/kd}$ mice

As $CoQ_{10}$ deficiency has been shown to cause impairment of $H_2S$ oxidation [18,33], we assessed effects of $CoQ_{10}$ supplementation on levels of the enzymes involved in the $H_2S$ oxidation pathway, sulfide quinone oxido-reductase (SQOR), thiosulfate sulfurtransferase (TST), persulfide dioxygenase (ETHE1) and sulfite oxidase (SUOX). We previously showed that in kidney of mutant 6 month-old mutant animals, levels of all the enzymes in this pathway are down-regulated [18].

Although in kidney of 6 month-old animals treated with $CoQ_{10}$, SQOR and SUOX proteins levels were lower than controls, the reduction was less pronounced than in placebo animals (protein levels Mut/WT ratios; kidney: SQOR: CoQ 6 mo 0.49 ± 0.16, $p < 0.0001$; SUOX: CoQ 6 mo 0.53 ± 0.16, $p < 0.0001$; Fig. 7A, D) while TST and ETHE1 levels were rescued (protein levels Mut/WT ratio; kidney: TST: CoQ 6 mo 0.80 ± 0.26; ETHE1: CoQ 6 mo 0.59 ± 0.12; Fig. 7B, C). However, in $CoQ_{10}$ treated 20 month–old mutants the levels of the four enzymes of the pathway showed no statistical difference compared to the $CoQ_{10}$ treated wild-type animals (Fig. 7). These results indicate that the rescue of $H_2S$ oxidation defect is one of the first and lasting effects of $CoQ_{10}$ supplementation.

To assess whether impairment of the sulfides oxidation pathway occurs early in CoQ deficiency, we measured protein levels of the enzymes in 1 month-old mutant mice. We found that SQOR levels were already significantly decreased compared with controls, while the levels of the downstream enzymes were comparable with wild type animals (protein levels Mut/WT ratio; kidney: SQOR: placebo 1 mo 0.79 ± 0.23, $p = 0.0056$; Fig. 7).

We previously showed that the mRNA levels of enzymes of the $H_2S$ oxidation were not decreased in kidney of 6 month untreated mutant animals. As expected, these levels were not affected by $CoQ_{10}$ supplementation (Suppl. Fig. 4).

In brain, $H_2S$ oxidation pathway in 6 month-old mutant animals was comparable with wild-type, before and after $CoQ_{10}$ supplementation (Suppl. Fig. 5). These findings indicate that decreased protein levels of SQOR, together with increased ROS production, comprise one of the first abnormalities occurring in kidney of *Pdss2*$^{kd/kd}$ mutant mice. Furthermore, they confirm our previous observation that CoQ levels regulate SQOR protein levels, which, in turn, modulate the downstream pathway [18].

### 3.8. $CoQ_{10}$ supplementation normalizes plasma acylcarnitine profile in *Pdss2*$^{kd/kd}$ mice

$H_2S$ accumulation inhibits short-chain acyl-CoA dehydrogenase (SCAD), leading to a defect of short-chain fatty acid oxidation [18,42–44]. Thus, we assessed acylcarnitine profile in blood of 6 month-old *Pdss2*$^{kd/kd}$ mice and we found increased AC-4 and AC-6 levels, and slightly increased AC-5 levels (acylcarnitine levels Mut/WT ratio; plasma: AC-4: Mut placebo 6 mo 2.36 ± 0.67, $p = 0.036$; AC-5: Mut placebo 6 mo 1.67 ± 0.52, $p = 0.14$; AC-6: Mut placebo 6 mo 2.28 ± 1.12, $p = 0.036$; Fig. 8A), which were normalized after $CoQ_{10}$ supplementation (Fig. 8A). These findings provide further evidence that $CoQ_{10}$ supplementation rescues $H_2S$ oxidation.

### 3.9. $CoQ_{10}$ supplementation rescues total GSH in kidney of *Pdss2*$^{kd/kd}$ mice

Because defects in $H_2S$ oxidation may decrease GSH levels through several mechanisms [21], we measured total GSH levels in kidney of treated and untreated animals. In fact, we observed that GSH levels were reduced in kidney of 6 month-old mutant mice [18] (Fig. 8B), and were rescued by $CoQ_{10}$ supplementation (total GSH levels Mut/WT ratio; kidney: Mut placebo 6 mo 0.71 ± 0.18, $p = 0.032$; Mut CoQ 6 mo 1.19 ± 0.21, $p = 0.19$; Fig. 8B). We also noted decreased GSH levels in pre-symptomatic stage of disease in kidney of 1 month-old mutant animals (total GSH levels Mut/WT ratio; kidney: Mut placebo 1 mo 0.64 ± 0.156, $p = 0.016$; Fig. 8B). These data indicate that low levels of GSH contribute to the pathogenesis of CoQ deficiency-associated NS.

### 3.10. Knock-down of SQOR in COQ8A depleted HeLa cells reduces antioxidant efficacy of $CoQ_{10}$

To evaluate whether the antioxidant effect of $CoQ_{10}$ is mediated by modulation of SQOR protein levels, we compared effects of $CoQ_{10}$ supplementation on levels of ROS in HeLa cells depleted of COQ8A, a CoQ biosynthesis regulatory protein and either with or without knock-down of SQOR transcript. COQ8A depleted cells, which have ~50% residual $CoQ_{10}$, showed increased ROS levels compared to control cells (160% of untreated EV, $p < 0.0001$; Fig. 9A, B). As expected, SQOR knock-down in control cells (55% of untreated EV, $p = 0.0165$; Fig. 9C) significantly increased levels of ROS (184% of untreated EV, $p < 0.0001$; Fig. 9A, B). In COQ8A depleted cells, $CoQ_{10}$ supplementation reduced ROS levels (62% of untreated CoQ8A depleted cells,

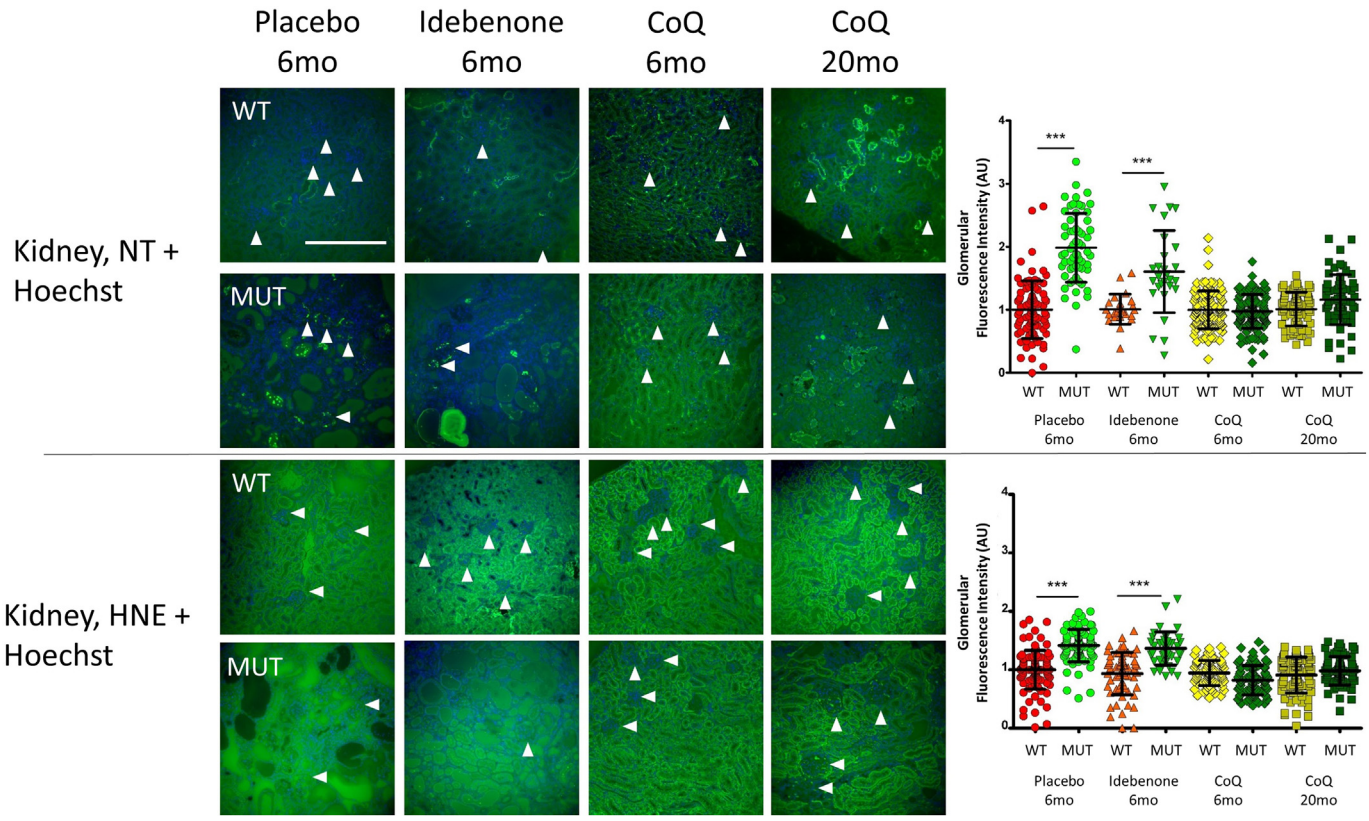


**Fig. 6.** Oxidative stress in kidney of *Pdss2*$^{kd/kd}$ mice: $CoQ_{10}$ supplementation improves oxidative stress. Representative images of anti-Nitrotyrosine and anti Hydroxynonenal staining to detect protein and lipid oxidation, and Hoechst to detect nuclei in mutant and control animals (N = 3 for group). White arrows indicate single glomeruli. Magnification: 20×; NT = Nitrotyrosine; HNE = 4 Hydroxynonenal; WT = wild type; Mut = mutant; CoQ = $CoQ_{10}$; 6 mo = 6 month old; 20 mo = 20 month old; scale bar = 100 μm. The graphics show the fluorescence intensity in the glomeruli. Data are represented as scatter plot measurements of single glomeruli fluorescence after background subtraction. Bars represent mean ± standard deviation. *** = $p < 0.001$ (One-way ANOVA with Tukey Post test). A.U. = Arbitrary Unit.

$p < 0.0001$; Fig. 9A, B) and increased SQOR protein levels (134% of untreated CoQ8A depleted cells, $p < 0.0317$; Fig. 9C) However, knockdown of SQOR in COQ8A depleted cells, prevented antioxidant effects of $CoQ_{10}$ supplementation (Fig. 9A, B, C). These data indicate that the antioxidant effects of $CoQ_{10}$ are mediated by the regulation of SQOR levels.

### 3.11. $CoQ_{10}$ is not metabolized in kidney of *Pdss2*$^{kd/kd}$ mice

$CoQ_{10}$ supplementation failed to completely rescue levels of $CoQ_{10}$ in kidney of mutant animals. Because exogenous $CoQ_{10}$ administered intraperitoneally to rat has been shown to be metabolized in kidney and excreted as metabolites in urine [2], we performed an ultra-carrying out liquid chromatography-mass spectrometer assay of urine from 6 month-old mutant and wild-type animals, with placebo or $CoQ_{10}$ supplementation. However, we were not able to detect any $CoQ_{10}$-derived metabolites (data not shown).

Lipidomics analysis of kidney and liver from 6 month-old *Pdss2*$^{kd/kd}$ mice also did not reveal $CoQ_{10}$ metabolites (data not shown). However, we found that short and medium length acylcarnitines levels showed trends toward decreases in both tissues (Fig. 10A, B). Diacylglycerol and triacylglycerol were reduced in both tissues (diacylglycerol, kidney: Mut pl 29% of WT, $p = 0.15$; liver: Mut pl 38% of WT, $p < 0.008$; triacylglycerol, kidney: Mut pl 0.27% of WT, $p = 0.4$; liver: Mut pl 0.43% of WT, $p < 0.008$; Table 4, Fig. 10C, D).

Lastly, cholesterol esters were increased in the mutant animals, and normalized by $CoQ_{10}$ supplementation (kidney: Mut pl 239%, $p = 0.28$; Mut CoQ 94% of WT, $p = 1$; liver: Mut pl 305%, $p < 0.016$; Mut CoQ 76% of WT, $p = 0.055$; Table 4, Fig. 10E, F). These results suggest that CoQ deficiency alters fatty acids oxidation, and cholesterol metabolism.

## 4. Discussion

This is the first study of a mouse model of primary $CoQ_{10}$ deficient nephrotic syndrome that links impairment of sulfide metabolism to oxidative stress and therapeutic effects of long-term oral $CoQ_{10}$ supplementation. Our results indicate that: 1) $H_2S$ oxidation impairment causes $CoQ_{10}$ associated-NS; 2) decreased SQOR levels increase ROS production and contributes to oxidative stress in CoQ deficiency; and 3) long-term $CoQ_{10}$ supplementation increases kidney CoQ levels sufficiently to rescue $H_2S$ oxidation by increasing SQOR levels thereby preventing renal failure.

Although previous studies explored the effects CoQ supplementation in *Pdss2*$^{kd/kd}$ mice, they were short-term studies, which showed improvement of NS, but did not elucidate the mechanism by which $CoQ_{10}$ supplementation rescues kidney function [17,45].

We previously showed that the increase of ROS levels is a kidney-specific phenomenon, and occurs in pre-symptomatic stage of disease, whereas impairment of CoQ-dependent respiratory chain activities is a late event [16]. Here, we show that abnormalities in the $H_2S$ metabolism pathway are also early events, occurring in pre-symptomatic stage of disease. We have observed that $CoQ_{10}$ supplementation rescues survival and prevents kidney failure in mutant animals, but does not correct respiratory chain enzymes activities. On the contrary, $CoQ_{10}$ administration improves oxidative stress and rescues $H_2S$ oxidation proportionally to the duration of the supplementation and increases mitochondrial mass. These results support the hypothesis that $H_2S$ oxidation impairment and oxidative stress together contribute to the

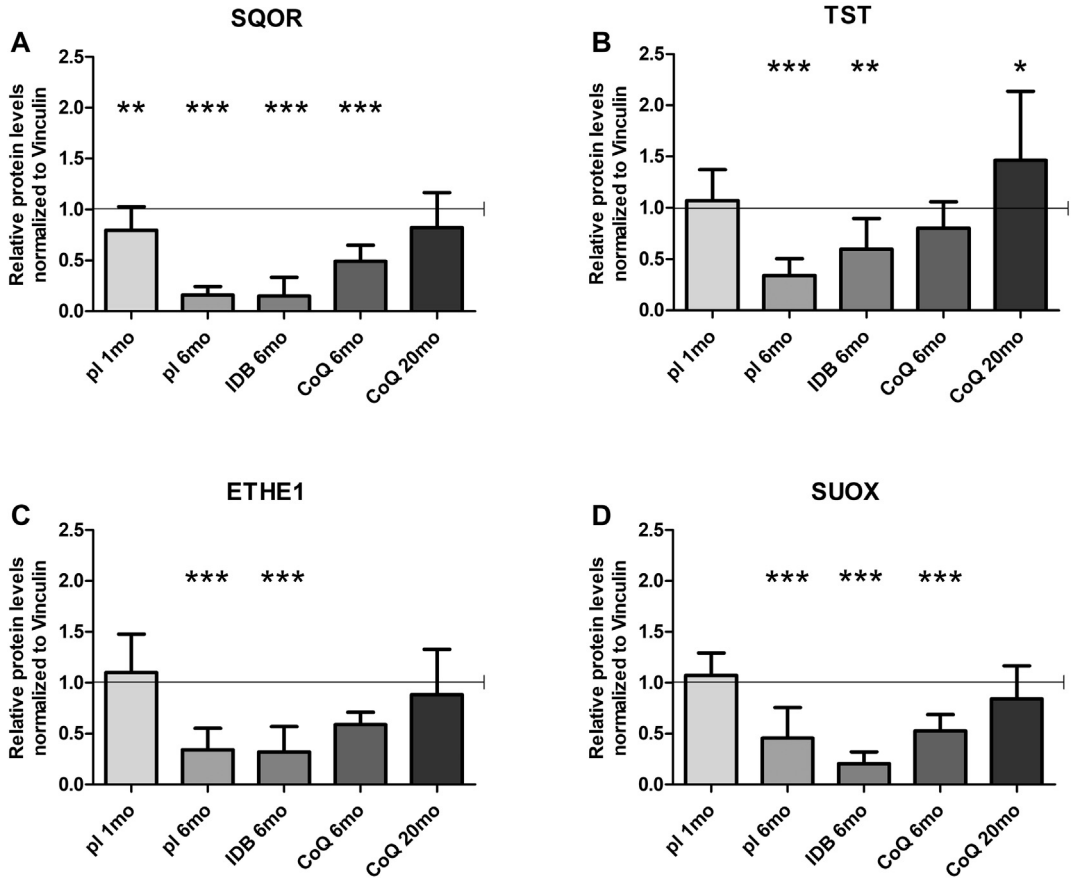


**Fig. 7.** Protein levels of the enzymes of the H2S oxidation pathway in kidney of *Pdss2*$^{kd/kd}$ mice: $CoQ_{10}$ supplementation rescues H2S oxidation impairment. Protein amounts of SQOR (A), TST (B) ETHE1 (C) and SUOX (D) normalized to vinculin and represented as fold changes of mutant mice (Mut) compared to age-matched controls (WT) under same treatment (pl = placebo; CoQ = $CoQ_{10}$; IDB = Idebenone) (all set as 1, horizontal bar). 1 mo = 1 month-old, 6 mo = 6 month old; 20 mo = 20 month old. Data are represented as mean ± standard deviation (N = 5 per group). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (Mann Whitney test).

pathogenesis of NS in CoQ deficiency, while defects of mitochondrial respiratory chain enzymes activities are not detrimental.

The causal relationship between $H_2S$ oxidation impairment and oxidative stress is corroborated by our experiments *in vitro*. Knock-down of SQOR in wild-type HeLa cells increases ROS to levels comparable to $CoQ_{10}$ deficient cells. Importantly, in CoQ deficient cells, $CoQ_{10}$ antioxidant effects are associated with increased SQOR, and are prevented by its knocking-down.

The causative role of oxidative stress in the pathogenesis of CoQ deficiency is supported by several studies *in vivo* and *in vitro*. As previously observed in *Pdss2*$^{kd/kd}$ mutant mice, the onset of the diseases correlates with signs of increased oxidative stress in affected organs [16]. Moderate CoQ deficiency *in vitro* leads to increased ROS levels and oxidative stress, which correlate with cell death, and they are both rescued by $CoQ_{10}$ supplementation [46,47]. In contrast, severe $CoQ_{10}$ deficiency in patients' fibroblasts [46,48], wild-type cells treated with inhibitors of $CoQ_{10}$ biosynthesis [47], and CoQ-deficient murine cell lines [49] decreases mitochondrial respiration, without evidence of growth impairment or increased cell death.

Interestingly, we observed that kidney of *Pdss2*$^{kd/kd}$ mice does not have alteration of respiratory supercomplexes levels, as opposed to *Coq9 knock-in* mouse brain, the clinically affected organ, which showed higher levels of free complex III than complex I/III in supercomplexes [32,50]. In *Coq9 knock-in* mice, encephalopathy and respiratory chain activities defects in brain were ameliorated by supplementation with reduced $CoQ_{10}$ (ubiquinol-10) [51], supporting our hypothesis that different pathomechanisms are responsible for the clinical heterogeneity of the disease. This difference can be explained by the fact that some of the proteins involved in CoQ biosynthesis, including $COQ_9$, are organized in a complex (Q complex), and different molecular defects affecting the stability of these proteins variably affect stability of the CoQ biosynthetic complex [50], and, in turn, in the hypothesis of an interaction between Q biosynthetic complex and supercomplexes (Trevisson E, personal communication), the stability of the respiratory supercomplexes. However, COQ1, encoded by *PDSS1* and *PDSS2*, is not part of the Q complex; therefore, mutations in *PDSS2* do not alter the complex structure.

In support of the idea that NS is not caused by RC defects is the fact that NS is not common in mitochondrial respiratory chain deficiencies, whose more frequent manifestation of kidney dysfunction is proximal tubulopathy [52,53]. Nevertheless, NS can rarely occur in patients with the mitochondrial DNA m.3243A > G mutation, a frequent cause of MELAS [54], which has been associated with secondary CoQ deficiency [41].

We recently showed that CoQ deficiency *in vitro* and *in vivo* causes $H_2S$ oxidation abnormalities with consequent $H_2S$ accumulation [18,55]. $H_2S$ is a gasotransmitter with several physiological functions, but when accumulated, is toxic. Therefore, $H_2S$ levels are tightly regulated by its synthesis and catabolism pathways [56,57]. Kidney might be particularly vulnerable because it produces $H_2S$ not only through the transulfuration pathway (using L-cysteine), but also through the DAO/3-MST pathway, in which 3-MP is generated from D-cysteine by D-amino acid oxidase (DAO) [58,59]. DAO is richly expressed in the kidney and may generate more $H_2S$ than the L-cysteine pathway [60]. Interestingly, DAO is also highly expressed also in cerebellum [61,62], which is another organ frequently affected in human

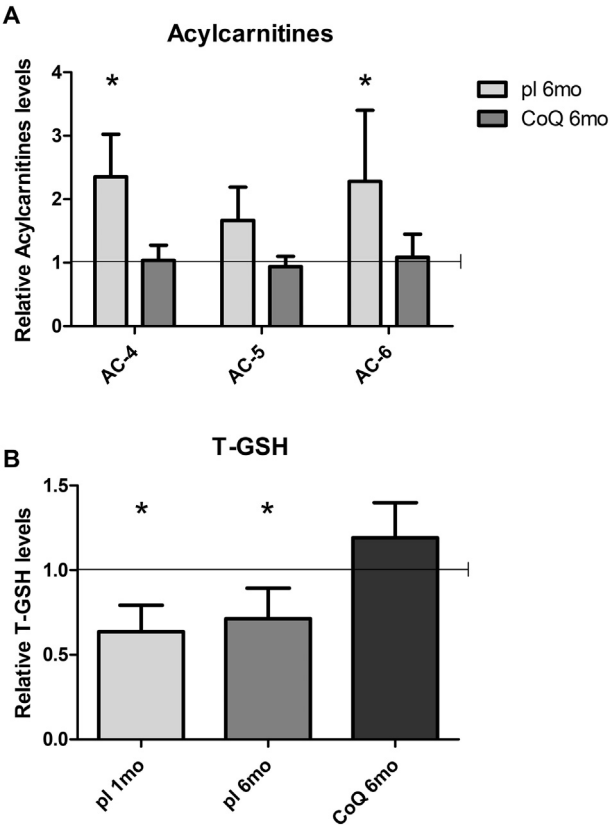


**Fig. 8.** Short-chain acylcarnitines in plasma and T-GSH in kidney of *Pdss2*$^{kd/kd}$ mice. $CoQ_{10}$ supplementation rescues detrimental effects of H2S oxidation impairment. Levels of C4, C5 and C6 acylcarnitines (A) and levels of total GSH (B) are represented as fold changes of mutant mice (Mut) compared to age-matched controls (WT) under same treatment (pl = placebo; CoQ = $CoQ_{10}$) (all set as 1, horizontal bar). 1 mo = 1 month-old, 6 mo = 6 month old, T-GSH = total glutathione. Data are represented as mean ± standard deviation (N = 5 for group). * = $p < 0.05$ (Mann-Whitney test).

$CoQ_{10}$ deficiency, primary and secondary [63,64]. Although *Pdss2*$^{kd/kd}$ mice do not manifest cerebellar ataxia, conditional cerebellar *Pdss2* knock-out mice develop cerebellar hypoplasia and ataxia [65].

However, other disorders of sulfide metabolism do not manifest glomerulonephropathy. For example, the levels of SQOR protein were significantly reduced in cerebrum, kidneys, and muscle of *Coq9R239X* mice, and in muscle of *Coq9Q95X* mice. In parallel to the reduction in SQOR protein levels, the activity of SQOR was significantly reduced in kidneys and muscle of *Coq9R239X* and *Coq9Q95X* mice [33]. Also ETHE1 knock out mice do not manifest nephropathy despite the high thiosulfate and $H_2S$ concentrations in kidney [66]. We have two possible explanations for the absence of nephropathy in these animals: 1) the severe encephalopathy causes early death and might mask the involvement of other organs, which would manifest later; 2) the phenotypic heterogeneity associated with different molecular defects might reflect tissue-specific vulnerability, compensatory mechanisms, alternative metabolic pathways for $H_2S$ detoxification, or different buffering mechanisms. Another difference between *Pdss2*$^{kd/kd}$ mice and ETHE1 knock out mice is the absence of COX deficiency in our model [18]. As we previously discussed [18], we observed a mild accumulation of $H_2S$ in mutant mice kidney compared with controls [18]. The only moderate increase in $H_2S$ concentrations we observed might explain the absence of COX deficiency in the affected tissues of the *Pdss2*$^{kd/kd}$ mice. It is possible that in CoQ deficiency, where SQOR activity is reduced but not completely absent, $H_2S$ levels are not high enough to suppress COX activity in contrast to ethylmalonic aciduria, which presents with a much more severe phenotype and COX deficiency in patients. However, kidney and liver of ETHE1 knock out mice, in spite of high thiosulfate and $H_2S$ concentrations, have normal COX activity [66], again supporting the hypothesis that there are tissue-specific alternative metabolic pathways for $H_2S$ detoxification, or different buffering mechanisms. Interestingly, *N*-acetylcysteine (NAC) supplementation in ETHE1 mice prolongs survival but does not improve COX activity in brain and muscle [67], suggesting other mechanisms of $H_2S$ toxicity, including inhibition of short-chain fatty acid oxidation, GSH depletion and the ability of $H_2S$ to generate ROS, may play a role in the pathogenesis of the disease.

Although the link between oxidative stress and $H_2S$ in kidney physiology and pathology is rather controversial due to conflicting data in this field, ROS production has been implicated as mechanism of $H_2S$ toxicity [20,21]. Since we excluded COX deficiency [16,18], we propose that low levels of GSH contribute to oxidative stress since we observed decreased GSH in kidney of 1 and 6 month-old *Pdss2*$^{kd/kd}$ mice, and GSH levels increased after $CoQ_{10}$ supplementation. Low levels of GSH may be a direct consequence of the down-regulation of the $H_2S$ catabolic pathway and its intermediates. Alternatively, GSH deficiency might be due to limited availability of its precursor cysteine, because $H_2S$ accumulation in kidney of *Pdss2*$^{kd/kd}$ mice might trigger negative feedback on the $H_2S$ synthesis pathway, which uses cysteine as substrate. Finally, since we previously observed that $CoQ_{10}$ deficiency *in vitro* causes increasing of S-sulfhydration of proteins involved in redox status of the cells [18], it is possible that this post-translational modification adversely affects their functions and contributes to oxidative stress in CoQ deficiency. In fact, protein S-sulfhydration has emerged as fundamental mechanism of $H_2S$ signaling [68,69].

It is plausible that oxidative stress in CoQ deficiency is not caused exclusively by defects of $H_2S$ oxidation; however, our experiments *in vitro* indicate that the antioxidant effects of CoQ are mediated by regulation of SQOR levels. Since CoQ receives electrons from NADH through complex I, $FADH_2$ through complex II, and $H_2S$ through SQOR [18], which is localized downstream of complex II and upstream of complex III, increased $H_2S$ oxidation induced by CoQ supplementation might decrease ROS generated by complex III. However, we cannot exclude that ROS originate directly from SQOR, since $H_2S$ oxidation has been shown to be coupled with oxygen consumption [69,70].

The severe deficiency of complexes I + III and II + III might be responsible for the renal tubular oxidative stress observed in 20 month-old animals. Interestingly, specific defects in the mitochondrial respiratory chain increase ROS levels, which have been implicated in prolonging rather than decreasing lifespan [71,72].

The lack of beneficial effects of IDB on oxidative stress in *Pdss2*$^{kd/kd}$ mice further indicates that $CoQ_{10}$ prevents oxidative stress by indirect mechanisms, since IDB is a synthetic analog of $CoQ_{10}$ with the same quinone moiety of $CoQ_{10}$, but with a shorter, less lipophilic tail, whose anti-oxidant properties, in a context of normal values of $CoQ_{10}$, are well known [22]. However, previous work in cultured fibroblasts and isolated mitochondria showed that IDB might have pro-oxidant properties under certain *in vitro* conditions [26,73]. The pro-oxidant effect of IDB is likely due to inhibition of complex I, as previously shown [24,25] and confirmed by the complex I + III defect observed in our animals treated with IDB.

Kidney, together with brain and muscle, has been described as one of the organs with poorest uptake of $CoQ_{10}$ [38], a data confirmed by two previous studies of short-term $CoQ_{10}$ supplementation in *Pdss2*$^{kd/kd}$ mice [17,45]. However, studies of long-term CoQ supplementation in wild-type animals showed accumulation of CoQ in kidney [74]. Our results not only confirm that long-term supplementation is necessary for $CoQ_{10}$ to reach target organs, but they also demonstrate that even when $CoQ_{10}$ reaches the target organ, not all of its biological functions are restored. It is noteworthy that SQOR and CoQ-dependent respiratory enzymes are both localized in the inner mitochondrial membrane and therefore should be equally reached by exogenous $CoQ_{10}$. Our results can be explained by the existence of two pools of CoQ, with separate

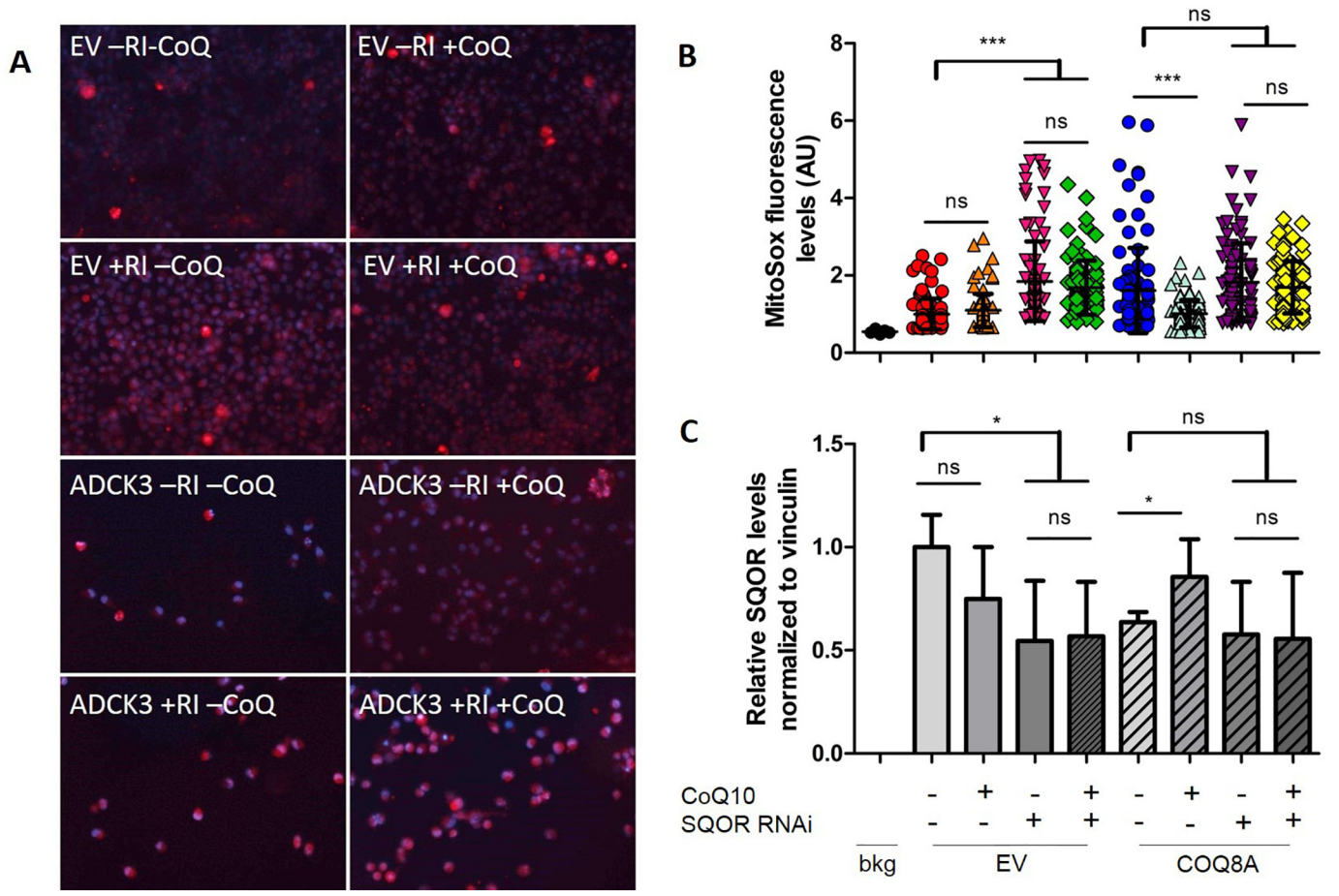


**Fig. 9.** Effects of $CoQ_{10}$ supplementation on ROS levels in SQOR and COQ8A depleted Hela cells: SQOR knock-down causes increase of ROS in wild-type cells and prevents antioxidant effects of $CoQ_{10}$ in COQ8A depleted cells. Representative images of HeLa cells stained with MitoSOX (red signal) to detect ROS, and Hoechst (blue signal) to detect nuclei (A). Quantification of red staining intensity. Data are represented as mean ± standard deviation (99 total readings from 3 independent experiments). ns = not significant; *** = $p < 0.001$ (One way ANOVA test) (B). Protein amounts of SQOR normalized to vinculin and represented as fold changes compared to untreated EV (C). Bkg = background; EV = empty vector; RI = *SQOR* RNA interference; CoQ = $CoQ_{10}$.

functions, one bound to mitochondrial respiratory chain supercomplexes [75], and a free pool in the inner mitochondrial membrane. Whether this free pool is merely a reservoir of an excess of CoQ molecules without a specific function, or the pool is necessary for the function of the respiratory chain and/or for additional functions is still debated [6,76,77]. We hypothesize that $CoQ_{10}$ oral supplementation increases the kidney free pool of CoQ, which might act as co-factor for SQOR, and not the CoQ bound to the supercomplexes. It is possible that $CoQ_{10}$ supplementation transiently increases CoQ bound to the supercomplexes, but in the attempt to reach the equilibrium with the free pool it diffuses, leading to the severe deficit of both CI + III and CII + III activities observed in 20 month-old mutant supplemented animals. An alternative hypothesis is that SQOR binding site is easier to access for CoQ than CI and CII binding sites, explaining why SQOR levels increase proportionally to the duration of the supplementation, while CI + III and CII + III are not affected by $CoQ_{10}$ supplementation. Another alternative hypothesis to explain how $CoQ_{10}$ rescues SQOR levels but not respiratory chain enzymes activities is that exogenous $CoQ_{10}$ can substitute $CoQ_9$ (the main specie of CoQ in mice) as electrons acceptor of SQOR, but not in the respiratory chain. However, this hypothesis is unlikely since $CoQ_{10}$ supplementation seems to equally increase $CoQ_9$ and $CoQ_{10}$ in kidney of *Pdss2*$^{kd/kd}$, likely *via* $CoQ_{10}$ conversion to $CoQ_9$ [74]. Importantly, in our study, CoQ supplementation did not affect SQOR transcriptional levels excluding the possibility that $CoQ_{10}$ accumulation in liver might trigger liver-kidney signaling pathways that induce *Sqor* mRNA up-regulation.

Although previous studies of short-term intra-peritoneal administration of radioactive $CoQ_{10}$ showed that exogenous $CoQ_{10}$ is metabolized in the kidney, and revealed urine metabolites [2], our metabolomics analyses did not detect any metabolites of ubiquinone in kidney or urine of treated and untreated wild-type and mutant animals. Furthermore, lipidomic analysis of kidney and liver also exclude the presence of $CoQ_{10}$-derived metabolites.

The lipidomic analysis also indicated that $CoQ_{10}$ deficiency alters cholesterol homeostasis. Specifically, mutant mice showed significant increases in cholesterol esters (CEs) containing long-unsaturated acyl chain, which might result from up-regulation in the uptake of cholesterol, or reduction in its efflux. Interestingly, and in contrast to liver, $CoQ_{10}$ supplementation rescued CEs levels in kidney, with a concomitant increase in the level of non-esterified cholesterol. In agreement with previous reports [78], this result suggests that $CoQ_{10}$ supplementation activates cholesterol metabolism, in a tissue-specific manner.

One limitation of the study is that biochemical and molecular abnormalities, beside oxidative stress, were measured in the whole kidney, and not specifically in the glomerulus, which is particularly vulnerable to *Pdss2* dysfunction. In fact, Peng and colleagues showed that disease manifestations originate specifically in glomerular podocytes, as renal disease is seen in podocytes conditional knockout mice but not in conditional knockouts targeted for example to renal tubular epithelium [27]. The same group reported evidence of mitophagy in *Pdss2*$^{kd/kd}$ mice [79]. Mitophagy was also reported in *in vitro* models of

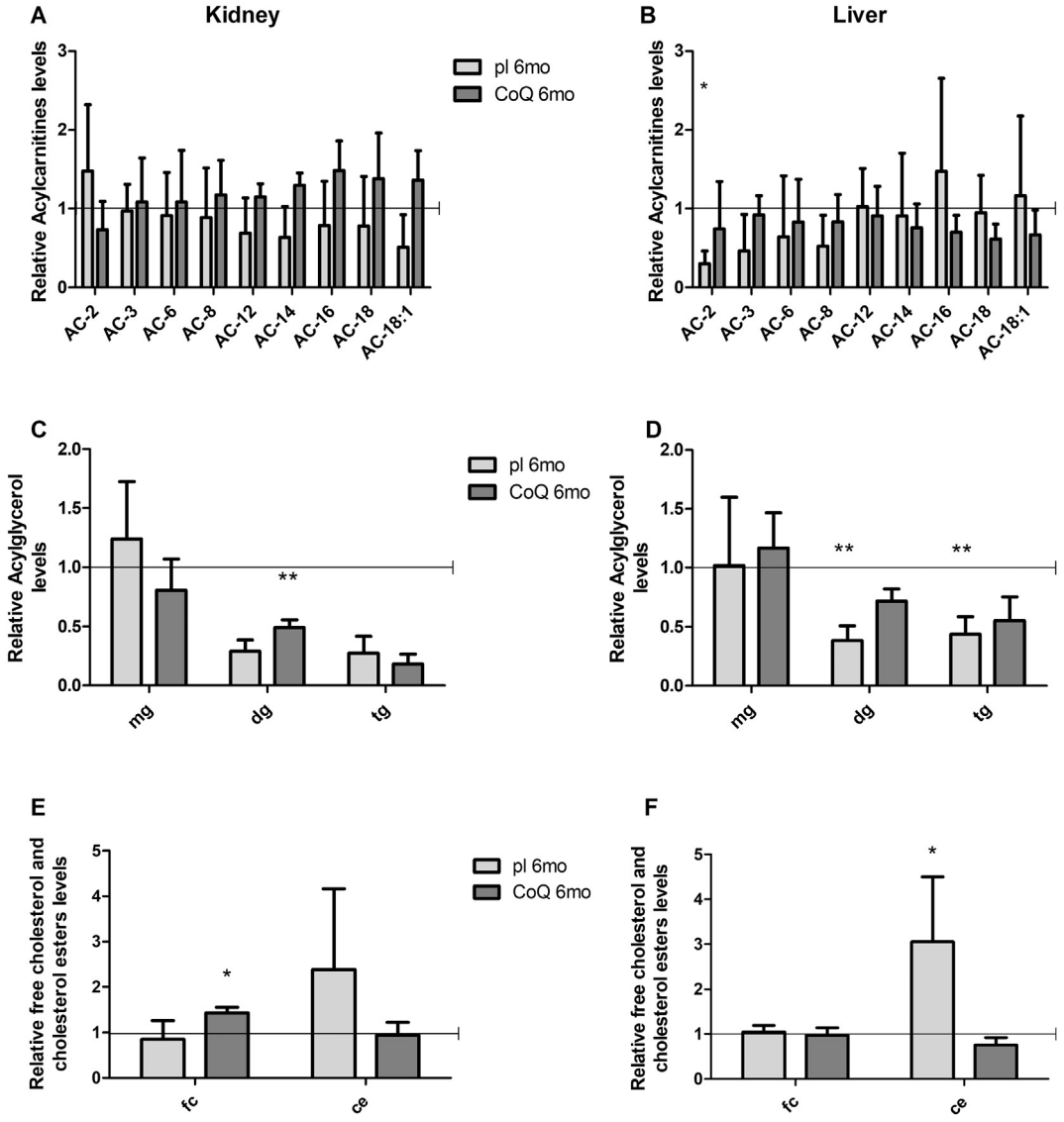


**Fig. 10.** Acylcarnitines, acylglycerols and cholesterols levels in kidney and liver of *Pdss2*$^{kd/kd}$ mice: CoQ deficiency leads to fatty acids and cholesterol metabolism alterations. Levels of acylcarnitines (A, B), acylglycerols (C, D) free cholesterol and cholesterol esters (E, F) in kidney (A, C, E) and liver (B, D, F) are represented as fold changes of mutant mice (Mut) compared to age-matched controls (WT) under same treatment (pl = placebo; CoQ = $CoQ_{10}$) (all set as 1, horizontal bar). 6 mo = 6 month old, AC = acylcarnitine, mg = monoacylglycerol, dg = diacylglycerol, tg = triacylglycerol, fc = free cholesterol, ce = cholesterol esters. Data are represented as mean ± standard deviation (N = 5 for group). * = $p < 0.05$, ** = $p < 0.01$ (Mann-Whitney test).

$CoQ_{10}$ deficiency [41]. We confirmed this data, which is in contrast with observations in patients with NS caused by *COQ2* mutations [10]. This discrepancy might be attributed to the rapid progression from onset of proteinuria to kidney failure is mice, which does not allow kidney proliferation of mitochondria as a transitory compensatory mechanism, as observed in humans.

In conclusion, our work further elucidates the role of $H_2S$ oxidation impairment in the pathogenesis of CoQ deficiency, and reveals a novel antioxidant mechanism of action of $CoQ_{10}$. These results not only are important to understand clinical heterogeneity and tissue-specificity of both primary and secondary CoQ deficiencies, but provide insight in a novel pathogenic pathway potentially relevant for other diseases.

## Author contributions

GK and EB performed the majority of the *in vivo* experiments; GK also wrote the manuscript; MZ performed the *in vitro* experiments, measured GSH *in vivo*, and quantified oxidative stress and kidney damage in mice; VE contributed to experiments of kidney morphology; AH-G performed the MS/MS; YX performed lipidomics analysis; CQ measured acylcarnitines profile; ST ran the HPLC; EA-G designed lipidomics experiment and analyzed results; LCL designed MS/MS experiment and analyzed results; CMQ designed the study, supervised the experiments, and wrote the manuscript. All authors critically reviewed the manuscript.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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The funders had no role in study design, data collection and

**Table 4**
Acylglycerols and cholesterols levels in kidney and liver of *Pdss2*$^{kd/kd}$ mice.

| | WT pl 6 mo | Mut pl 6 mo | WT CoQ 6 mo | Mut CoQ 6 mo |
|---|---|---|---|---|
| Kidney | | | | |
| mg | 1 ± 0.54 | 1.24 ± 0.48 | 1 ± 0.32 | 0.81 ± 0.26 |
| dg | 1 ± 1.16 | 0.29 ± 0.1 | 1 ± 0.48 | **0.49 ± 0.07**** |
| tg | 1 ± 1.05 | 0.27 ± 0.14 | 1 ± 0.66 | 0.18 ± 0.08 |
| fc | 1 ± 0.41 | 0.85 ± 0.41 | 1 ± 0.26 | **1.43 ± 0.12*** |
| ce | 1 ± 0.75 | 3.4 ± 2.74 | 1 ± 0.55 | 0.94 ± 0.28 |
| Liver | | | | |
| mg | 1 ± 0.35 | 1.02 ± 0.58 | 1 ± 0.28 | 1.17 ± 0.3 |
| dg | 1 ± 0.13 | **0.38 ± 0.13**** | 1 ± 0.32 | 0.72 ± 0.10 |
| tg | 1 ± 0.21 | **0.44 ± 0.15**** | 1 ± 0.59 | 0.55 ± 0.20 |
| fc | 1 ± 0.25 | 1.40 ± 0.15 | 1 ± 0.12 | 0.97 ± 0.17 |
| ce | 1 ± 0.19 | **3.06 ± 1.45*** | 1 ± 0.12 | 0.76 ± 0.16 |

Values are represented as fold changes of mutant group (Mut) compared to age-matched controls (WT) under same treatment. WT = wild type; Mut = mutant, mo = month-old; pl = placebo; CoQ = $CoQ_{10}$; mg = monoacylglycerol, dg = diacylglycerol, tg = triacylglycerol, fc = free cholesterol, ce = cholesterol esters; N = 5 per group. Bold characters = statistical significance.

* $p < 0.05$.

** $p < 0.01$. (Mann Whitney test).

analysis, decision to publish, or preparation of the manuscript.

## Conflict of interest statement

The authors have declared that no conflict of interest exists.

## Appendix A. Supplementary data

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