



**UNIVERSITY of THESSALY**  
**SCHOOL OF PHYSICAL EDUCATION & SPORT SCIENCE**  
DEPARTMENT OF PHYSICAL EDUCATION & SPORT SCIENCE



Doctor of Philosophy Dissertation

**«Mechanisms of muscle function: the effect of uremia on biochemical  
status and oxidative stress»**

by

**Konstantina P. Poulianiti**

Submitted in partial fulfillment  
of the requirements for the degree  
of Doctor of Philosophy  
Trikala, 2017



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## Acknowledgments

*Many people supported me during my PhD studies who deserve to be acknowledged: Committee members and supervisors, colleagues, friends and family who have routinely gone beyond their academic and collegiate duties to encourage me and who helped instill great confidence in both myself and my work.*

*Foremost I would like to express my deepest gratitude to the whole scientific team of the THALES EU funded project, which financially supported my PhD studies, especially the Scientific Coordinator Professor Yiannis Koutedakis, and the Principal Investigator Assoc. Professor Christina Karatzaferi, as well as the University of Thessaly' Research Committee.*

*I would like to express my special gratitude to my principal supervisor and scientific director of this thesis, Assoc. Professor Christina Karatzaferi, who led my three-member supervisory committee for the longest period, for giving me the opportunity and the guidance to study for my PhD degree, inspiring me with her knowledge, patience and motivation and especially introducing me into the wonders of physiology.*

*I would also like to thank Professor Athanasios Jamurtas for his expert advice, help, support every time I needed him, especially with regards to human biochemistry aspects.*

*I am grateful to Professor Yiannis Koutedakis for his expert advice but also for always finding solutions when administration or finances or other problems 'failed' us.*

*I am indebted to Professor Ioannis Stefanidis for overseeing key aspects of the experimental renal model development and advice on the thesis but also the overall collaboration with the Medical School.*

*My special appreciation is also given to Ass. Professor Giorgos Sakkas for his supervision and help during the whole project and for clarifying to me the clinical applications of this work.*

*I also thank my fellow lab mate Georgia Mitrou for her friendship, help and support. Everything would be very different if I had to go through all this process without her company.*

*I overall sincerely appreciate the cooperation with the “Muscle Lab” & “Live Lab” teams of Drs Karatzaferi and Sakkas, including Dr. Antonia Kaltsatou, Dr. Aggeliki Karioti, Dr Christina Malavaki, Dr Stefania Grigoriou, Giorgo Theofilidi, Argyro Krasse and others, including members of the ‘Biochemistry’ team and the ‘Fame Lab’ team leader Dr Andreas Flouris.*

*I thank them all for their help and collaboration, during various stages of this project and my overall academic development. It was a great experience to be collaborating on various scientific and academic goals, discussing new methods, setting up new assays, preparing for conferences and encouraging one another.*

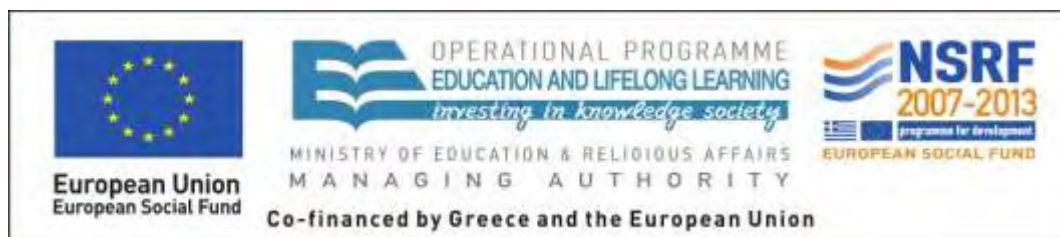
*Moreover I am grateful to all my friends for their friendship and support.*

*This dissertation is dedicated to my family and to Themistoklis Tsatalas for their love and support all these years of my studies.*

*Konstantina Poulianiti*

*Trikala 2017*

This research has in part been co-financed by the European Union (European Social Fund - ESF) and Greek national funds through the Operational Program "Educational and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Thales (MuscleFun Project-MIS 377260) Investing in knowledge society through the European Social Fund.



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## List of abbreviations

<b>AGE</b>	Advanced glycosylation end-product
<b>AOPPs</b>	Advanced oxidation protein products
<b>BCA</b>	Bicinchoninic acid
<b>CKD</b>	Chronic Kidney Disease
<b>CVD</b>	Cardiovascular Disease
<b>NADPH</b>	$\beta$ -Nicotinamide adenine di-nucleotide
<b>CAT</b>	Catalase
<b><math>\gamma</math>-GCS</b>	gamma-glutamylcysteine synthetase
<b>GSH</b>	Reduced form of Glutathione
<b>GSH-S</b>	Glutathione synthetase
<b>GPx</b>	Glutathione Peroxidase
<b>GSSG</b>	Oxidized form of Glutathione
<b>DCM</b>	Dichloromethane
<b>DPPH</b>	2,2-Diphenyl-1-picrylhydrazyl
<b>DNPH</b>	2,4-Dinitrophenylhydrazine
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>ESRD</b>	End Stage Renal Disease
<b>EPO</b>	Erythropoietin
<b>GFR</b>	Glomerular Filtration Rate
<b>Hb</b>	Hemoglobin
<b>HCl</b>	Hydrochloric acid
<b>HD</b>	Hemodialysis
<b>HDL</b>	High Density Lipoprotein
<b>PS</b>	Polysulfone
<b>HFR</b>	Haemodiafiltration
<b>Ht</b>	Hematocrit
<b>ID</b>	Iron dextran

<b>IS</b>	Iron Sucrose
<b>KDIGO</b>	Kidney Disease Improving Global Outcomes
<b>LDL</b>	Low Density Lipoprotein
<b>MHCf</b>	Myosin heavy chain
<b>MCH</b>	Mean Corpuscular Hemoglobin
<b>MCV</b>	Mean Corpuscular Volume
<b>MDA</b>	Malondialdehyde
<b>NAC</b>	N-acetylcysteine
<b>NaOH</b>	Sodium hydroxide
<b>NEM</b>	N-ethylmaleimide
<b>NO</b>	Nitric oxide
<b>NOX</b>	NADPH oxidases
<b>ORAC</b>	Oxygen radical absorbance capacity
<b>Ox-LDL</b>	Oxidized Low Density Lipoprotein
<b>PBS</b>	Phosphate Buffer Saline
<b>PD</b>	Peritoneal dialysis
<b>PON1</b>	Paraoxonase
<b>RBC</b>	Red Blood Cells
<b>ROS</b>	Reactive Oxygen Species
<b>SEM</b>	Standard error of the mean
<b>SFGC</b>	Sodium ferrigluconate complex, in sucrose
<b>SOD</b>	Superoxide Dismutase
<b>TAC</b>	Total Antioxidant Capacity
<b>TAS</b>	Total Antioxidant Status
<b>TBA</b>	2-Thiobarbituric acid
<b>TBARS</b>	Thiobarbituric Acid Reactive Substances
<b>TCA</b>	Trichloroacetic acid
<b>TEAC</b>	Trolox equivalent antioxidant capacity
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor- $\alpha$
<b>XO</b>	Xanthine oxidase
<b>O<sub>2</sub><sup>•-</sup></b>	Superoxide anion

<b>CO<sub>2</sub></b>	Carbon dioxide
<b>OH<sup>•</sup></b>	Hydroxyl radical
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>O<sub>2</sub></b>	Singlet oxygen
<b>ONOO<sup>-</sup></b>	Peroxynitrite
<b>8-OH-dG</b>	8-hydroxy-2'-deoxyguanosine

## Abstract

**Introduction:** Chronic Kidney Disease (CKD) is characterized as a silent epidemic and undoubtedly is one of the most severe chronic disease worldwide. It is accompanied by several comorbidities such as anemia, cardiovascular diseases, atherosclerosis and diabetes, which contribute to the reduced quality of life and to the mortality of the patients. Additionally CKD is accompanied by several skeletal muscle abnormalities, in part linked to hypokinesia and in part to uremic toxicity. Skeletal muscle's weakness and metabolic imbalance, are components of functional and morphological alterations which are collectively termed uremic myopathy. It is suggested that oxidative stress heavily contribute to all the above pathologies encountered in CKD. Although the progression of CKD is associated with the harmful effects of oxidative stress, its role has not yet been fully clarified during pre-dialysis stages of the disease.

**Aims:** The aim of this study, using an animal model of CKD, was to evaluate the systemic effects of CKD in redox status indices as reflected in blood levels (Research Paper 1). Moreover, the study evaluated the skeletal muscle specific effects of uremia on muscle redox status. Specifically two different types of skeletal muscle, the fast psoas and the slow soleus were analyzed (Research Paper 2). Also, the biochemical and hematological parameters in our early CKD model and its possible association with oxidative stress levels were evaluated (Research Paper 3). Last but not least it was examined whether the oxidative stress markers measured in blood adequately reflected redox status in skeletal muscle and whether redox status evaluated in psoas muscle reflected the redox status in soleus. (Research Paper 4).

**Methods:** We used an animal model (partial nephrectomy) of renal disease in New Zealand white female rabbits. Surgery and euthanasia (after 3 months) protocols were approved by the ethic committee of the University of Thessaly. Renal insufficiency was induced by removal of the right kidney and partial nephrectomy of the left kidney (Uremic group, N=9). Control animals underwent sham operation (Control group, N=6). From both groups, blood and skeletal muscle (psoas, soleus) samples were collected and analysed for the Reduced Glutathione (GSH), Oxidized Glutathione

(GSSG), GSH/GSSG Ratio, Total Antioxidant Capacity (TAC), Thiobarbituric Acid Reactive Substances (TBARS) and Protein Carbonyls (PC) levels and for GR-Reductase and Catalase (CAT) activities. Blood samples were also analyzed for the biochemical (lipid profile) indices Total Cholesterol, High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Triglycerides, Triglycerides/HDL Ratio Glucose and Bilirubin and for the hematological parameters leukocytes, red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH). Urea, creatinine, and plasma total protein levels were also evaluated.

**Results:** (Research Paper 1) GSH concentration was significantly higher in the Uremic group ( $17.50 \pm 1.73 \mu\text{mol/g protein}$ ) compared to Control ( $12.43 \pm 1.01 \mu\text{mol/g protein}$ ),  $p=0.033$ . TBARS concentration tended to be higher in the Uremic group ( $7.03 \pm 0.81 \text{ nmol/ml}$ ), compared to Control ( $5.12 \pm 0.42 \text{ nmol/ml}$ ),  $p=0.060$ . No significant differences were found in the rest of redox status indices evaluated in blood of Control and Uremic groups ( $p>0.05$ ).

(Research Paper 2) Significant group main effects were found for PC in both muscle types with its concentration to be higher in the Uremic group (psoas:  $1.086 \pm 0.294$ , soleus:  $2.52 \pm 0.29 \text{ nmol/mg protein}$ ) compared to the Control (psoas:  $0.596 \pm 0.372$ , soleus:  $0.929 \pm 0.41 \text{ nmol/mg protein}$ ). Significant muscle main effects were found for Total protein, PC, TBARS, GSH, TAC, CAT and GR-Reductase activity in both the Control and the Uremic group. Total protein and TAC concentrations were significantly lower in soleus compared to psoas muscle in both the Control and the Uremic group. Additionally, soleus demonstrated higher levels of TBARS and PC levels as well as higher GSH levels, Catalase and GR-Reductase activities compared to psoas muscle in both the Control and Uremic group. No interactions were found between the two examined muscle types (psoas, soleus) and two groups (Control, Uremic) for all the redox status indices ( $p>0.05$ ).

(Research Paper 3) We found no significant changes in the blood lipid profile of the Uremic group as compared to the Control group ( $p>0.05$ ). However, the ratio of Triglycerides/HDL was 5.67, much higher than the Control group value, at 4.63. Moreover, RBC, Hematocrit and MCH were found to be significant decreased in the Uremic group ( $3.92 \pm 0.29 \times 10^6/\text{mm}^3$ ,  $26.06 \pm 1.85 \%$ ,  $19.76 \pm 0.67 \text{ pg/cell}$ ) as



compared to the Control ( $5.10 \pm 0.17 \times 10^6/\text{mm}^3$ ,  $35.24 \pm 0.79 \%$ ,  $21.64 \pm 0.44$  pg/cell),  $p < 0.05$ , and MCV had a tendency to be lower in the Uremic group ( $65.40 \pm 1.89 \text{ mm}^3$ ) as compared to the Control group ( $69.78 \pm 1.36 \text{ mm}^3$ ),  $p = 0.087$ .

(Research Paper 4) For each one of the redox status indices, statistical analysis didn't show any significant correlation between blood and the two examined muscles' levels neither in the pool of samples nor for each group separately ( $p > 0.05$ ). Regarding the correlation analysis between Urea, Creatinine and redox status in blood and skeletal muscle, while no relationship reached statistical significance it is noteworthy that in the pool data Creatinine levels tended to correlate with blood GSH ( $r = 0.664$ ,  $p = 0.073$ ). Likewise, Urea tended to correlate with blood GSH ( $r = 0.655$ ,  $p = 0.078$ ). Although in the Control group there was no significant correlation in redox status indices between the two different types of skeletal muscle ( $p > 0.05$ ), in the Uremic group there was significant correlation between psoas and soleus muscle in PC levels ( $r = 0.913$ ,  $p = 0.002$ ), in GSSG levels ( $r = 0.766$ ,  $p = 0.027$ ) levels and in CAT levels ( $r = 0.743$ ,  $p = 0.035$ ) respectively.

**Discussion:** In the current PhD Thesis an increase in blood GSH levels of the Uremic group was found probably as an adaptive response to keep redox homeostasis stable. Indeed the tendency for increased lipid peroxidation (TBARS) constitutes an early sign of oxidative stress appearance in blood of our model of pre-dialysis stage of CKD. This is probably indicative of a premature development of cardiovascular problems and atherogenesis in pre dialysis patients (Research Paper 1). In addition increased PC levels in both muscle types of the Uremic group were observed. This is regarded as a very interesting finding for the time point of protein oxidation since it reveals the early emergence of oxidative damage to protein content. Regarding the two different types of skeletal muscle, soleus demonstrated higher TBARS levels, irrespective of group, consistent to the differences in mitochondrial content, as soleus has higher amount thus is more exposed to ROS. However we observed that soleus muscle developed an appropriate antioxidant capacity to withstand the oxidative load. Despite the reduced TAC levels in soleus muscle in comparison with psoas, the increased levels GSH, CAT and GR-reductase indicate that healthy soleus indeed demonstrates a higher antioxidant capacity than psoas, with uremic soleus appearing to have likewise further up-regulated its defenses (Research Paper 2). Regarding lipid profile, perhaps more time was needed for our model to develop disturbances since no

changes were found between the two groups. However the tendency for increased plasma lipid peroxidation (TBARS) and for a higher Triglycerides/HDL ratio in the Uremic group fits well with a progressive atherogenic development due to CKD. On the other hand, an impairment in the hematological profile of the Uremic group was observed which constitutes early signs of anemia development (Research Paper 3). The current study also observed that there is no correspondence between blood and skeletal muscle redox status indices in CKD, demonstrating that tissue specific redox status changes do not cause corresponding changes that can be measured in blood. It is possible that factors like the animal model, the techniques differences and the stage of the disease may mask muscle's contribution to blood levels of redox indices in our data. The significant correlations of redox status indices among the two types of skeletal muscle that were found only for the Uremic group, indicated that the experimentally induced CKD posed an appreciable redox challenge for skeletal muscle, whose samples presented with a coordinated variability in redox status levels (Research Paper 4).

**Conclusions:** In conclusion, the results of this work demonstrate that even in the pre-dialysis stages of CKD there is an emergence of oxidative stress in blood and as an adaptive response the antioxidant defense mechanisms are upregulated in order to preserve redox status homeostasis. Moreover in both types of skeletal muscle (psoas, soleus) carbonyl formation indicates an early stimulus of muscle protein degradation. However both oxidative damage and antioxidant response seem to be muscle type-specific. Our model of CKD shows that the lipid profile in this stage of the disease development does not seem to be disturbed to a great extent. However, an atherogenic development cannot be excluded and should be addressed early on. Still, we found a severely disturbed hematological profile in experimental animals. This highlights that early recognition of anemia, in pre-dialysis stage, could play a key role in its treatment and could probably delay the progression of CKD. Last but not least, it was found that blood levels of the redox status indices did not reflect muscle concentrations and more work is needed in the direction of less invasive monitoring of muscle related redox imbalances. Overall, our results highlight the need for interventions early during disease progression in order to protect skeletal muscle quality, maintain redox balance and correct anemia, and thus allow patients to reach the end-stage at the best possible status.

## Περίληψη

**Εισαγωγή:** Η Χρόνια Νεφρική Νόσος (XNN) χαρακτηρίζεται ως μία σιωπηλή επιδημία και αναμφίβολα αποτελεί μία από τις πιο σοβαρές ασθένειες παγκοσμίως. Η ασθένεια συνοδεύεται από παθήσεις, όπως είναι η αναιμία, τα καρδιαγγειακά νοσήματα, η αθηροσκλήρωση και ο διαβήτης, οι οποίες συμβάλλουν στην μειωμένη ποιότητα ζωής και τη θνησιμότητα των ασθενών. Επιπρόσθετα, η XNN συνοδεύεται από διάφορες παθήσεις των σκελετικών μυών που εν μέρει αποδίδονται στην υποκινητικότητα των ασθενών αλλά και στην ουραιμική τοξικότητα. Η αδυναμία του σκελετικού μυός καθώς και οι μεταβολικές διαταραχές του αποτελούν στοιχεία λειτουργικών και μεταβολικών αλλοιώσεων τα οποία συγκεντρωτικά ονομάζονται ουραιμική μυοπάθεια. Έχει προταθεί ότι το οξειδωτικό στρες συμβάλει σε μεγάλο βαθμό σε όλες τις παραπάνω παθολογικές καταστάσεις που εμφανίζονται στη XNN. Αν και η εξέλιξη της νόσου σχετίζεται με τις επιβλαβείς επιδράσεις του οξειδωτικού στρες, ο ρόλος του δεν έχει πλήρως διαλευκανθεί σε προγενέστερα στάδια της νόσου πριν το τελικό στάδιο (αιμοκάθαρση).

**Σκοπός:** Ο σκοπός της μελέτης ήταν να αξιολογήσει τις συστηματικές επιδράσεις της XNN στο οξειδοαναγωγικό προφίλ του αίματος, χρησιμοποιώντας ένα ζωικό μοντέλο προ-τελικού σταδίου XNN (Μελέτη 1). Επίσης, στόχος ήταν να αξιολογηθεί η επίδραση της XNN στο οξειδοαναγωγικό προφίλ του σκελετικού μυός. Συγκεκριμένα εξετάστηκαν δύο διαφορετικοί τύποι σκελετικού μυός, ο ψοΐτης (ταχείας συστολής) και ο υποκνημίδιος (βραδείας συστολής) (Μελέτη 2). Η μελέτη επίσης αξιολόγησε το βιοχημικό και αιματολογικό προφίλ του ζωικού μοντέλου και εξετάστηκε η πιθανή συσχέτιση τους με το οξειδωτικό στρες (Μελέτη 3). Τέλος, εξετάστηκε κατά πόσο οι δείκτες οξειδωτικού στρες που αξιολογήθηκαν στο αίμα αντικατοπτρίζουν επαρκώς την οξειδοαναγωγική κατάσταση στο μυ και αν η οξειδοαναγωγική κατάσταση που μετρήθηκε στον ψοΐτη μυ αντανακλά την οξειδοαναγωγική κατάσταση του υποκνημιδίου (Μελέτη 4).

**Μεθοδολογία:** Χρησιμοποιήθηκε ένα ζωικό μοντέλο XNN και συγκεκριμένα λευκοί θηλυκοί κόνικλοι Νέας Ζηλανδίας. Τα πρωτόκολλα της εγχείρισης και της ευθανασίας (μετά από 3 μήνες) εγκρίθηκαν από την Επιτροπή Βιοηθικής του Πανεπιστημίου Θεσσαλίας. Η νεφρική ανεπάρκεια προκλήθηκε με την αφαίρεση του

δεξιού νεφρού και την μερική νεφρεκτομή του αριστερού νεφρού (ουραιμική ομάδα N=9). Η ομάδα ελέγχου υποβλήθηκε σε εικονική εγχείριση (ομάδα ελέγχου, N=6). Και από τις δύο ομάδες ζώων συλλέχθηκαν δείγματα αίματος και σκελετικού μυός (ψοίτης, υποκνημίδιος) και προσδιορίστηκαν οι συγκεντρώσεις της Ανηγμένης Γλουταθειόνης (GSH), της Οξειδωμένης Γλουταθειόνης (GSSG), του λόγου GSH/GSSG, της Ολικής Αντιοξειδωτικής Ικανότητας (TAC), των Ουσιών που αντιδρούν με Θειοβαρβιτουρικό Οξύ (TBARS), των Πρωτεϊνικών Καρβονύλιων (PC) καθώς και οι δραστηριότητες της Ρεδοκτάσης της γλουταθειόνης (GR – Reductase) και της Καταλάσης (CAT). Στα δείγματα του αίματος προσδιορίστηκαν επίσης οι συγκεντρώσεις των βιοχημικών δεικτών όπως η Ολική Χοληστερόλη, η Υψηλής-Πυκνότητας Λιποπρωτεΐνη (HDL), η Χαμηλής-Πυκνότητας Λιποπρωτεΐνη (LDL), τα Τριγλυκερίδια, ο λόγος Τριγλυκερίδια/HDL, η Γλυκόζη και η Χολερυθρίνη καθώς και τα επίπεδα των αιματολογικών παραμέτρων όπως τα Λευκοκύτταρα, τα Ερυθρά αιμοσφαίρια (RBC), η Αιμοσφαιρίνη (Hb), ο Αιματοκρίτης (Hct), ο Μέσος όγκος ερυθρών (MCV) και η Μέση περιεκτικότητα αιμοσφαιρίνης (MCH). Μετρήθηκαν επίσης η Ουρία, η Κρεατινίνη και η Ολική πρωτεΐνη πλάσματος.

**Αποτελέσματα:** (Μελέτη 1) Η συγκέντρωση της GSH βρέθηκε σημαντικά υψηλότερη στην ουραιμική ομάδα ( $17.50 \pm 1.73 \mu\text{mol/g protein}$ ) σε σχέση με την ομάδα ελέγχου ( $12.43 \pm 1.01 \mu\text{mol/g protein}$ ),  $p=0.033$ . Η συγκέντρωση των TBARS παρουσίασε μία τάση για αύξηση στην ουραιμική ομάδα ( $7.03 \pm 0.81 \text{ nmol/ml}$ ), σε σχέση με την ομάδα ελέγχου ( $5.12 \pm 0.42 \text{ nmol/ml}$ ),  $p=0.060$ . Δεν βρέθηκαν άλλες σημαντικές διαφορές στους υπόλοιπους δείκτες οξειδωτικού στρες που μετρήθηκαν στο αίμα των δύο ομάδων ( $p>0.05$ ).

(Μελέτη 2) Σημαντική επίδραση της ομάδας παρατηρήθηκε για τα PC και στους δύο μύες με την συγκέντρωσή τους να είναι υψηλότερη στην ουραιμική ομάδα (ψοίτης:  $1.086 \pm 0.294$ , υποκνημίδιος:  $2.52 \pm 0.29 \text{ nmol/mg protein}$ ) σε σχέση με την ομάδα ελέγχου (ψοίτης:  $0.596 \pm 0.372$ , υποκνημίδιος:  $0.929 \pm 0.41 \text{ nmol/mg protein}$ ). Σημαντική επίδραση του μυός παρατηρήθηκε για τις συγκεντρώσεις των Total protein, PC, TBARS, GSH, TAC, και της δραστηριότητας των CAT και GR-Reductase και στις δύο ομάδες με τα επίπεδά τους να είναι υψηλότερα στην ουραιμική ομάδα (ψοίτης:  $1.086 \pm 0.294$ , υποκνημίδιος:  $2.52 \pm 0.29 \text{ nmol/mg protein}$ ) σε σχέση με την

ομάδα ελέγχου (ψοΐτης:  $0.596 \pm 0.372$ , υποκνημίδιος:  $0.929 \pm 0.41$  nmol/mg protein). Η συγκεντρώσεις των Total Protein και TAC ήταν σημαντικά χαμηλότερες στον υποκνημίδιο μυ σε σχέση με τον ψοΐτη και στις δύο ομάδες. Επίσης ο υποκνημίδιος παρουσίασε υψηλότερα επίπεδα TBARS και PC όπως επίσης και υψηλότερα επίπεδα GSH, Catalase και GR-Reductase σε σχέση με τον ψοΐτη τόσο στην ουραιμική όσο και στην ομάδα ελέγχου. Δεν παρατηρήθηκαν σημαντικές αλληλεπιδράσεις μεταξύ των δύο τύπων μυός και των δύο ομάδων για όλους τους δείκτες οξειδωτικού στρες ( $p > 0.05$ ).

(Μελέτη 3) Δεν υπήρξαν σημαντικές αλλαγές στο λιπιδαιμικό προφίλ της ουραιμικής ομάδας συγκριτικά με την ομάδα ελέγχου ( $p > 0.05$ ). Ωστόσο ο λόγος Τριγλυκερίδια/HDL βρέθηκε 5,67, πολύ υψηλότερος από την τιμή 4 της ομάδας Ελέγχου. Επίσης οι συγκεντρώσεις των RBC, Hematocrit και MCH βρέθηκαν σημαντικά χαμηλότερες στην ουραιμική ομάδα ( $3.92 \pm 0.29 \times 10^6/\text{mm}^3$ ,  $26.06 \pm 1.85$  %,  $19.76 \pm 0.67$  pg/cell) σε σχέση με την ομάδα ελέγχου ( $5.10 \pm 0.17 \times 10^6/\text{mm}^3$ ,  $35.24 \pm 0.79$  %,  $21.64 \pm 0.44$  pg/cell),  $p < 0.05$ , και το MCV παρουσίασε μία τάση για μείωση στην ουραιμική ομάδα ( $65.40 \pm 1.89$   $\text{mm}^3$ ) σε σχέση με την ομάδα ελέγχου ( $69.78 \pm 1.36$   $\text{mm}^3$ ,  $p = 0.087$ ).

(Μελέτη 4) Για κάθε έναν από τους δείκτες οξειδωτικού στρες που αξιολογήθηκε, η στατιστική ανάλυση δεν έδειξε καμία σημαντική συσχέτιση μεταξύ των επιπέδων του στο αίμα και των επιπέδων του στους δύο τύπους μυός τόσο στο σύνολο των δεδομένων (pool data) όσο και σε κάθε ομάδα ξεχωριστά ( $p > 0.05$ ). Επίσης, ενώ δεν παρατηρήθηκε σημαντική συσχέτιση στα επίπεδα Ουρίας και Κρεατινίνης και των δεικτών του οξειδοαναγωγικού προφίλ στο αίμα και στο σκελετικό μυ, αξίζει να σημειωθεί ότι στο σύνολο των δεδομένων (pool data), τα επίπεδα της Κρεατινίνης παρουσίασαν μία τάση για συσχέτιση με τα επίπεδα της GSH στο αίμα ( $r = 0.655$ ,  $p = 0.078$ ). Αν και στην ομάδα ελέγχου δεν υπήρξε σημαντική συσχέτιση των δεικτών οξειδοαναγωγικού προφίλ μεταξύ των δύο διαφορετικών τύπων μυός ( $p > 0.05$ ), στην ουραιμική ομάδα παρατηρήθηκε σημαντική συσχέτιση μεταξύ ψοΐτη και υποκνημίδιου αναφορικά με τα επίπεδα των PC ( $r = 0.913$ ,  $p = 0.002$ ), της GSSG ( $r = 0.766$ ,  $p = 0.027$ ) και της CAT ( $r = 0.743$ ,  $p = 0.035$ ).

**Συζήτηση:** Στην παρούσα διδακτορική διατριβή παρατηρήθηκε μία αύξηση στα επίπεδα της GSH στο αίμα της ουραιμικής ομάδας κονίκλων, που πιθανότατα

αποτελεί έναν προσαρμοστικό μηχανισμό απόκρισης στο οξειδωτικό στρες με σκοπό τη διατήρηση της οξειδοαναγωγικής ομοιόστασης. Πράγματι υπήρχε μία τάση για αύξηση του δείκτη λιπιδικής υπεροξειδωσης (TBARS) γεγονός που αποτελεί ένα πρώιμο σημάδι αυξημένου οξειδωτικού στρες στο αίμα του ουραιμικού μοντέλου. Φαίνεται δηλαδή να υπάρχει ή έναρξη της εκδήλωσης ορισμένων αιτιών πρόκλησης καρδιαγγειακών προβλημάτων και αθηρογένεσης στους ασθενείς με ΧΝΝ (Μελέτη 1). Επιπλέον διαπιστώθηκαν αυξημένα επίπεδα των πρωτεϊνικών καρβονυλίων και στους δύο τύπους μυϊκού ιστού στην ομάδα των ουραιμικών κουνελιών. Αυτό μπορεί να θεωρηθεί ως ένα πολύ ενδιαφέρον εύρημα για τη χρονική στιγμή που λαμβάνει χώρα η πρωτεϊνική οξείδωση αφού φανερώνει μία πρώιμη εκδήλωση βλάβης σε επίπεδο πρωτεϊνών. Όσον αφορά τους δύο διαφορετικούς τύπους σκελετικού μύος, στον υποκνημίδιο διαπιστώθηκαν υψηλότερα επίπεδα TBARS, ανεξαρτήτως της ομάδας. Το αποτέλεσμα αυτό οποίο είναι σύμφωνο με το διαφορετικό αριθμό των μιτοχονδρίων ανάμεσα στους δύο μύες, με τον υποκνημίδιο να παρουσιάζει υψηλότερο αριθμό και συνεπώς να είναι περισσότερο εκτεθειμένος σε ROS. Ωστόσο διαπιστώθηκε πως ο υποκνημίδιος μύς ανέπτυξε παράλληλα μία έντονη αντιοξειδωτική δραστηριότητα ώστε να αντιμετωπίσει τα αυξημένα επίπεδα του οξειδωτικού στρες. Παρόλο που τα επίπεδα TAC ήταν μειωμένα στον υποκνημίδιο μυ σε σύγκριση με τον ψοϊτή, τα αυξημένα επίπεδα GSH, CAT and GR-reductase υποδεικνύουν πως σε υγιείς συνθήκες ο υποκνημίδιος δύναται να αναπτύξει αυξημένη αντιοξειδωτική ικανότητα σε σύγκριση με τον ψοϊτή και σε συνθήκες ουραιμίας αυτή η ικανότητα αυξάνεται ακόμη περισσότερο (Μελέτη 2). Σχετικά με το λιπιδαιμικό προφίλ, πιθανόν να απαιτούνταν περισσότερος χρόνος για ουραιμικό μοντέλο ώστε να αναπτυχθούν διαταραχές αφού καμία διαφορά δεν βρέθηκε ανάμεσα στις ομάδες. Ωστόσο, η τάση που παρατηρήθηκε για αυξημένη λιπιδική υπεροξειδωση (TBARS) στο πλάσμα και η υψηλότερη τιμή του λόγου Τριγλυκερίδια/HDL στην ουραιμική ομάδα αποτελούν ένα σημάδι εμφάνισης προβλημάτων αθηρογένεσης εξαιτίας της ασθένειας. Αναφορικά με το αιματολογικό προφίλ της ουραιμικής ομάδας, παρατηρήθηκε μια σημαντική διαταραχή σε σχέση με την ομάδα ελέγχου γεγονός που αποτελεί πρώιμο σημάδι εμφάνισης της αναιμίας (Μελέτη 3). Στην παρούσα μελέτη παρατηρήθηκε επίσης ότι στο μοντέλο της ΧΝΝ δεν υπήρξε συσχέτιση μεταξύ των οξειδοαναγωγικών δεικτών που μετρήθηκαν στο αίμα με αυτούς που μετρήθηκαν στους δύο τύπους μύος, αποδεικνύοντας οι οξειδοαναγωγικές αλλαγές που συντελούνται στο μυ, δεν αντανακλώνται στις

αντίστοιχες μετρήσεις που διεξάγονται στο αίμα. Παράγοντες όπως, το ζωικό μοντέλο, οι διαφορές μεταξύ των τεχνικών μέτρησης των δεικτών και το στάδιο της ασθένειας είναι πιθανόν να κάλυψαν τη συμβολή του μυός στα επίπεδα οξειδωτικού στρες στο αίμα. Οι σημαντικές συσχετίσεις που παρατηρήθηκαν μόνο στην ουραιμική ομάδα σχετικά με τα επίπεδα των δεικτών οξειδοαναγωγικού στρες μεταξύ των δύο διαφορετικών τύπων μυός, υποδεικνύουν ότι η πειραματικά προκαλούμενη νεφρική ανεπάρκεια αποτελεί μία αξιόλογη πρόκληση για τη μελέτη του οξειδοαναγωγικού προφίλ του σκελετικού μυός, του οποίου τα δείγματα παρουσιάζουν μία συντονισμένη μεταβλητότητα στα επίπεδα οξειδοαναγωγικής κατάστασης.

**Συμπεράσματα:** Τα ευρήματα της παρούσας μελέτης υποδηλώνουν ότι ακόμη και στα μη τελικά στάδια (αιμοδιάλυση) της ΧΝΝ το οξειδωτικό στρες εμφανίζεται στο αίμα και σαν απόκριση προσαρμογής η αντιοξειδωτική άμυνα αυξάνεται με σκοπό να διατηρήσει σταθερή την οξειδοαναγωγική ομοιόσταση. Επίσης, και στους δύο τύπους μυός (ψοίτη, υποκνημίδιο) ο σχηματισμός πρωτεϊνικών καρβονυλίων υποδεικνύει ένα πρώιμο ερέθισμα αποσύνθεσης των μυϊκών πρωτεϊνών. Ωστόσο, τόσο η οξειδωτική βλάβη όσο και η αντιοξειδωτική απόκριση φαίνεται να εξαρτώνται από τον τύπο του μυός. Επίσης, στο λιπιδαιμικό του προφίλ του μοντέλου ΧΝΝ φάνηκε ότι δεν υπήρξαν σημαντικές διαταραχές σε αυτό το στάδιο της ασθένειας. Δεν μπορεί ωστόσο, να αποκλειστεί εντελώς η πρώιμη εμφάνιση αιτιών αθηρογένεσης η οποία και πρέπει να αντιμετωπιστεί από νωρίς. Ακόμη, βρέθηκε σημαντική διαταραχή στο αιματολογικό προφίλ του μοντέλου, υποδηλώνοντας ότι η έγκαιρη αναγνώριση της αναιμίας σε μη τελικό στάδιο, μπορεί να παίξει πολύ σημαντικό ρόλο στην θεραπεία της και στην πρόοδο της ασθένειας. Τέλος, όπως διαπιστώθηκε, τα επίπεδα των οξειδοαναγωγικών δεικτών στο αίμα δεν αντικατοπτρίζουν την εικόνα της οξειδοαναγωγικής κατάστασης μέσα στο μυ και για το λόγο αυτό χρειάζεται περισσότερη μελέτη στην κατεύθυνση της λιγότερο επεμβατικής παρακολούθησης των οξειδοαναγωγικών ανισορροπιών τους μυός.

Συνολικά, τα αποτελέσματα υπογραμμίζουν την ανάγκη για έγκαιρες παρεμβάσεις, στα πρώιμα στάδια της νόσου με σκοπό την προστασία της ακεραιότητας των σκελετικών μυών, τη διατήρηση της οξειδοαναγωγικής ισορροπίας και την διόρθωση της αναιμίας, επιτρέποντας στους ασθενείς να φτάσουν στο τελικό στάδιο της νόσου σε όσο το δυνατόν καλύτερη κατάσταση.

# 1. Introduction



Chronic kidney disease (CKD) is described as a complex state, where the kidney progressively loses its function and during this pathological condition a variety of metabolic abnormalities occurs, influencing several organs of the human body (Kovesdy & Kalantar-Zadeh, 2009a), including skeletal muscle. When kidney loses a significant part of its function, the terms “chronic renal failure” or “chronic kidney disease” are used to describe this condition. If less than 10% of kidney function remains, then the disease is usually referred to as “end stage renal disease” (ESRD) (McCance & Huether, 2002).

Whether at a pre-dialysis stage or at the end-stage, as the disease progresses, CKD patients experience symptoms of muscle weakness, early fatigue, exercise intolerance, and present with signs such as muscle atrophy, metabolic dysregulation, insulin resistance, fat infiltration, all linked to the disturbance of skeletal muscle quantity and quality, collectively termed ‘uremic myopathy’ (Campistol, 2002). Several interventions, including while helpful, can’t fully remedy the pathological phenotype. Muscle loss and weakness contribute to the high morbidity and mortality of these patients, especially at the end-stage of the disease. Complex mechanisms that stimulate muscle dysfunction in CDK have been proposed and oxidative stress could clearly be implicated given that skeletal muscle itself contributes notably in redox balance (Powers, Ji, Kavazis, & Jackson, 2011). Recent data have revealed that 2.6 million people receive dialysis treatment all over the world and by 2030 around 5.4 million people are expected to receive renal replacement therapy. Taking these numbers into consideration, it can be easily recognized that CKD has become a global public health problem (García-García & Jha, 2015).

In recent years, only in the United States, the annual cost for patients suffering from end-stage renal disease (ESRD), has reached \$40 billion. Regarding the patients receiving dialysis therapy, the annual cost is three times higher than the expenditures for the patients who have received kidney transplantation ("Kidney and Urologic Diseases Statistics for the United States," 2010). In the last 30 years a large increase in the number of deaths due to ESRD has been also noticed, from 10,478 in 1980 to 90,118 in 2009. In parallel, not so precisely ascertained but present nevertheless is the cost that the disease impacts on life enjoyment and mental health. Such patients experience an extremely low Quality of Life linked to low physical activity levels and the severe comorbidities of CKD (such as cardiovascular disease, diabetes and other) (Ayus, Frommer, & Young, 1981; Sakkas, Ball, et al., 2003).

As far as patients' life expectancy is concerned, the 5-year survival rate for transplant patients is 85.5%, while the same rate for dialysis patients is almost the half, 35.8% ("Kidney and Urologic Diseases Statistics for the United States," 2010). Many factors are implicated in the high mortality of these patients, with CVD being the leading cause of death (Gosmanova & Le, 2011).

There is strong evidence suggesting that CKD is accompanied by an enhanced state of oxidative stress, which in turn is associated with higher risk of developing cardiovascular disease (CVD), morbidity and mortality (Bayes, Pastor, Bonal, Foraster, & Romero, 2006). The presence of oxidative stress in CKD patients is manifested by increases in the levels of several oxidative damage markers and by a parallel reduction in their antioxidant defense capacity (Stenvinkel & Barany, 2002; Terawaki et al., 2004). This state of redox imbalance is linked to additional pathological complications that CKD patients present with, such as malnutrition, anemia, and cardiovascular disease including atherosclerosis (Grune, Sommerburg, & Siems, 2000; Macdougall & Cooper, 2002; Stenvinkel & Barany, 2002). However most studies evaluating redox disturbances in CKD patients have been performed in blood and have focused on the end-stage (Kaltsatou et al., 2015) leaving unanswered questions as to the situation at the level of skeletal muscle, the influence of fiber type and the overall development of oxidative stress in pre-dialysis stages.

Generally, a variety of mechanisms have been implicated in the generation of oxidative stress in CKD, such as antioxidant deficiency, chronic inflammation (Hensley, Robinson, Gabbita, Salsman, & Floyd, 2000; Zimmermann, Herrlinger, Pruy, Metzger, & Wanner, 1999) including advanced glycation mediated disturbances of glomerular homeostasis as disease progresses (Yamagishi et al., 2002). In addition, for the end-stage patients (ESRD), neutrophil activation during hemodialysis (HD) therapy (Grooteman et al., 1997) and dialysis treatment *per se* contribute to the increase of oxidative damage observed in patients with CKD (Miyazaki et al., 2000; Morena, Cristol, & Canaud, 2000; Nourooz-Zadeh, 1999). Four main factors have been proposed to be responsible for the harmful effects of oxidative stress in patients under HD : the uremic milieu, the HD treatment *per se*, the hemoincompatibility of dialysis system and the concomitant drug treatment (Canaud et al., 1999).

In pre-dialysis patients the situation is less studied. One reason is that not only is CKD 'silent' in its early stages, but also that patients are led to CKD following

different paths (some from hypertension, some from glucose intolerance, others from nephrotic symptoms, (Yamagata et al., 2007) with oxidative stress not figuring as yet as high in the priority for treatment. Moreover, the relatively few animal studies so far (McLeland, Cianciolo, Duncan, & Quimby, 2015; Silva et al., 2013; Zhao, Niu, Zhang, Hou, & Du, 2009) have been either dealing with acute kidney damage or other mechanisms. A chronic animal model, allowing for the development of renal insufficiency and maintenance of the animal at a pre-dialysis stage (controlling for diet and gender) would be more appropriate to study oxidative stress in chronic renal insufficiency independently from confounding factors which plague patient studies.

Until today most studies examining the redox status in CKD patients focused on the end stage of the disease when pathology is already too advanced or irreversible. Additionally, few studies have focused on the redox status evaluation of skeletal muscle with most focusing on blood. However, it remains unclear when during disease progression the redox imbalance manifests itself, and which one of its components, defense capacity or ROS overproduction, becomes critical first.

## **2. Aims – Significance**

The primary aim of the current PhD research thesis was to investigate the effects of uremia on biochemical status and oxidative stress tolerance by using an animal model of CKD. More specifically the aims of this research were:

1. To investigate the effects of uremia on blood redox status of uremic rabbits as compared with controls (sham-operated) counterparts.
2. To investigate the effects of uremia on muscle redox status in two different types of skeletal muscle, (psoas and soleus) of uremic rabbits as compared with controls (sham-operated) counterparts.
3. To assess the effects of uremia on biochemical status in both blood and muscle (psoas and soleus) of uremic rabbits as compared with controls (sham-operated) counterparts.
4. To investigate a possible relationship between the changes in blood and muscle redox status between the two groups as a result of uremia.

### 3. Literature Review

Part of the following Literature Review has appeared in Poulianiti et al., 2016, Systemic Redox Imbalance in Chronic Kidney Disease: A systematic review. *Oxid Med Cell Longev*, doi: 10.1155/2016/8598253, IF:4.492 and Kaltsatou et al., Uremic myopathy: is oxidative stress implicated in muscle dysfunction in uremia? *Frontiers in Physiology*, *Front Physiol*. doi: 10.3389/fphys.2015.00102, 2015 Mar 30;6:102, IF:4.031.

### 3.1 Renal failure and CKD

According to KDIGO 2012 ("KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease,") CKD is defined as the manifestation of abnormalities in kidney structure or function, being present for more than 3 months and accompanied by several implications in health. CKD is classified, according to the Glomerular Filtration Rate (GFR category) or albuminuria levels (albuminuria category CGA). The stages of CKD are mainly based on the measured or estimated GFR. Five stages of the disease exist, where kidney function is regarded as normal in stage 1 and is progressively reduced from stages 2 to 5.

- Stage 1: GFR  $\geq$  90 ml/min – Normal or high kidney function
- Stage 2: GFR 60-89 ml/min – Mildly decreased kidney function
- Stage 3a: GFR 45-59 ml/min – moderately decrease kidney function
- Stage 3b: GFR 30-44 ml/min – moderate decrease kidney function
- Stage 4: GFR 15-29 ml/min – severely decrease kidney function
- Stage 5: GFR  $<$  15 ml/min – kidney failure (requiring kidney transplantation or dialysis to maintain life)

CKD is accompanied by uremia, a clinical syndrome which is characterized by a multitude of changes in the metabolic, fluid and electrolyte balance of the organism. Piorry was the first to use the term uremia in order to describe this complex condition, which emerges when kidney function deteriorates (Piorry & l'Heritier, 1840).

In uremic syndrome, urea and other metabolic waste products are retained in blood and tissues, due to kidney dysfunction and its incapability to normally excrete them through urine. Metabolic waste products are substances left over from metabolic processes (cellular respiration, protein metabolism, etc.) which cannot be used by the organism and therefore, they should be excreted. These includes nitrogen compounds, like ammonia, urea and uric acid, as well as water, CO<sub>2</sub>, phosphates, sulfates, etc.

Uremia is accompanied by various symptoms such as fatigue, nausea, loss of appetite, neurological dysfunction, vomiting, pruritis and increased levels of blood urea and creatinine. As far as renal failure progress occurs and renal clearance is being continuously reduced, several compounds, molecules and solutes tend to accumulate in blood and other tissues of the uremic organism. Until today, no single uremic toxin has been identified to be responsible for all the clinical manifestations of uremia. A variety of toxins such as beta-2-microglobulin, parathyroid hormone (PTH), polyamines, advanced glycosylation end products, indoxyl sulfate and other middle molecules are thought to play a role in the uremic syndrome (Yavuz et al., 2005). More recently the progression of technology helped to the identification of many new uremic retention solutes such as adipate, malonate, methylmalonate,  $\alpha$ -phenylacetyl-L-glutamine, 5-hydroxyindole, indoxyl glucuronide, using proteomic and metabonomic methods (Aronov et al., 2011; Rhee et al., 2010). Furthermore, there is no specific mechanism which causes the uremic milieu, but a multifactorial process takes place including retained solutes, imbalance of significant hormones and metabolic disorders (McCance & Huether, 2002).

### **3.1.1 Blood manifestations of CKD**

Several biochemical manifestations take place due to the uremic syndrome and the eventual progression of renal failure. Hyperphosphatemia, hypokalemia/hyperkalemia (Lowrie & Lew, 1990) and hyperparathyroidism (Ganesh, Stack, Levin, Hulbert-Shearon, & Port, 2001) have been observed as adverse outcomes in CKD and have been associated with increased morbidity and mortality of patients. Anemia is a common characteristic in CKD including a combination of factors such as erythropoietin (EPO) deficiency (Besarab & Ayyoub, 2007a), deterioration or inhibition of erythropoiesis process, due to the uremic environment (J. W. Eschbach, 1989), disturbed iron homeostasis (Babitt & Lin, 2010) and short red blood cell life (Vos et al., 2011).

Another common feature of patients with CKD is protein-energy wasting (PEW), which constitutes one of the greatest risk factors associated with negative consequences and mortality (Kovesdy & Kalantar-Zadeh, 2009b). This term has been established by the International Society of Renal Nutrition and Metabolism (ISRNM)



in order to describe the “state of decreased body stores of protein and energy fuels”. Protein-energy waste is associated with increased dysfunctionality that emerges from metabolic abnormalities (Fouque et al., 2008). An imbalance in a variety of biomarkers such as creatinine (Lowrie & Lew, 1990), transferrin (Kalantar-Zadeh et al., 1998), serum albumin (Beddhu et al., 2002), prealbumin (Sreedhara et al., 1996) cholesterol (Iseki, Yamazato, Tozawa, & Takishita, 2002), and hormones like thyroid hormones (Zoccali, Mallamaci, Tripepi, Cutrupi, & Pizzini, 2006), leptin (Scholze, Rattensperger, Zidek, & Tepel, 2007), visfatin (Axelsson et al., 2007) and adiponectin (Zoccali et al., 2002) has been demonstrated to be responsible for the protein energy wasting in CKD. Apart from the above imbalances, insulin resistance is also a common feature in patients with CKD, suggesting that disturbance in insulin signaling may also play an important role in protein waste (Sakkas, Karatzaferi, et al., 2008).

It is considered that inflammation strongly affects patients with CKD and increased levels of inflammatory and proinflammatory cytokines have been observed in plasma, such as IL-1 $\beta$ , IL-6, IL-8 TNF-a, C-reactive protein and serum amyloid A (Zhang et al., 2009; Zhang et al., 2011). Two of these increased levels of cytokines, IL-1 $\beta$  and TNF-a are not confined only in hemodialyzed patients, but also in pre dialysis stage (Pereira et al., 1994).

### **3.1.2 Skeletal muscle\_ Structure and function**

Skeletal muscle is one of the most dynamic and plastic tissues in the human body, comprising approximately of 40% of total body weight and containing 50-75% of all body proteins. Muscle is mainly composed of water (75%), protein (20%) and other substances such as minerals, fat, inorganic salts and carbohydrates (5%) and contributes to multiple body functions (Frontera & Ochala, 2015).

The main function of skeletal muscle is to convert chemical energy into mechanical energy generating force and power, to maintain posture and to produce movement. From a metabolic point of view, skeletal muscle contributes to basal energy metabolism, being storage for significant substrates such as carbohydrates and amino acids, the production of heat for the maintenance of core temperature, and the consumption of oxygen and energy used during physical activity (American College of Sports Medicine, 2012; Frontera & Ochala, 2015; Wolfe, 2006).

The architecture of skeletal muscle is characterized by an arrangement of muscle fibers which are also referred to as myofibers or muscle cells, surrounded by connective tissue. The significant variability of biochemical, metabolic and mechanical phenotypes of individual skeletal muscle fiber enables muscle to respond with flexibility during activities with various metabolic and mechanical demands.

During the last few decades, muscle fibers have been classified using different criteria. The most frequently used classification for adult human limb muscles includes the: (1) color of muscle fibers (red vs. white) depending on myoglobin content, (2) speed of shortening during a single twitch (fast vs. slow), (3) predominance of certain metabolic or enzymatic pathways (oxidative vs. glycolytic), (4) degree of fatigability during sustained activation (fatigable vs. fatigue-resistant), (5) protein isoform expression such as myosin heavy chain (MCH). Thus, three fiber types are commonly referred: type I (slow-twitch, red, oxidative, fatigue-resistant), IIA (fast-twitch, red, oxidative, intermediate metabolic properties, fatigue resistant) and IIX (fast-twitch, white, glycolytic, fatigable) (Wolfe, 2006). However, it has been demonstrated that more than one type of MCH can be expressed simultaneously, for example type I and IIA, or IIA and IIX together creating the so-called hybrid fibers which have been shown to increase with aging, exercise and under pathological conditions (American College of Sports Medicine, 2012; Andersen, 2003).

### **3.1.3 Skeletal muscle related manifestations in CDK**

It has been reported that uremic myopathy is common with an overall prevalence of 50 % in dialysis patients (Clyne, 1996), usually appears when GFR is under 25ml/min and its progression parallels the decline of renal function (Campistol, 2002)



**Figure 3.1.** The multifactorial nature of uremic myopathy. Many specific disease-related but also lifestyle factors contribute to the pathological muscle state (Figure appearing in (Kaltsatou et al., 2015)).

Although the pathogenesis of uremic myopathy has not been yet clarified, uremic toxicity and hypokinesia seem to play a key role for these muscle abnormalities in patients with CKD and especially in ESRD patients undergoing hemodialysis (HD) therapy. A significant correlation between GFR and exercise tolerance has been observed, revealing the low activity and functionality of CKD patients (Clyne, Jogestrand, Lins, & Pehrsson, 1994; Clyne, Jogestrand, Lins, Pehrsson, & Ekelund, 1987). A variety of interventions, such as exercise has been introduced in order to treat or stop muscle deterioration in pre-dialysis (Clyne, Ekholm, Jogestrand, Lins, & Pehrsson, 1991) and dialysis patients (Johansen et al., 2006; Sakkas, Sargeant, et al., 2003). Despite the evident improvement in exercise capacity and muscle morphology (Sakkas, Hadjigeorgiou, et al., 2008; Sakkas, Sargeant, et al., 2003), in increasing muscle mass with steroid supplements (Topp et al., 2003), in improving sleep and overall quality of life (Sakkas, Karatzaferi, et al., 2008), none of the interventions can restore muscle functionality in ESRD patients to the level of age-matched healthy sedentary individuals (Sakkas, Hadjigeorgiou, et al., 2008; Sakkas, Sargeant, et al., 2003). Furthermore morphological abnormalities have been noticed in locomotory as well as in non-locomotory muscles of CKD patients

(Sakkas, Ball, et al., 2003), leading to the conclusion that not all of the dysfunction can be attributed to hypokinesia.

Apart from the compromised or accelerated muscle waste, the decreased ability to anabolize muscle could also be an issue in CKD. Interventions with nandrolone decanoate has positive effects on increasing muscle mass but no improvement in muscle strength was observed (Topp et al., 2003). In the study of Zhang *et al* (Zhang, Wang, Wang, Du, & Mitch, 2010) a delayed regeneration of damaged muscle and reductions in MyoD protein was observed in a mouse model of CKD, indicating a decreased satellite cell proliferation and differentiation.

As it is mentioned above, CKD patients are characterized by muscle loss and weakness. It has been also demonstrated that this waste of muscle strength is a factor for easy fatigability in these patients and can be associated with loss of muscle fibers and atrophy of the remaining fibers (Sakkas, Ball, et al., 2003),(Porter, Vandervoort, & Lexell, 1995). Abnormal mitochondrial respiratory capacity and disturbed mitochondrial morphology can also be referred as additional factors for premature fatigue in CKD patients (Kouidi et al., 1998).

#### **3.1.4 Oxidative stress**

The majority of molecular species maintain the electrons in their outer orbital, arranged in pairs, which is fundamental for their stability and function. A free radical is defined as an atom or molecule that contains one or more single unpaired electron in the outer orbital. This structure of unpaired electron makes the radical unstable and highly reactive, thus it reacts with other molecules in order to pair this electron and become stable (Guertens, De Boeck, Highley, van Oosterom, & de Bruijn, 2002). In 1954, Commoner *et al* first reported that biological material produces free radicals (Commoner, Townsend, & Pake, 1954).

Free radicals can be generated in cells during several reactions, by losing or gaining a single electron. The general term reactive oxygen species (ROS) includes not only the oxygen-centered radicals but also the non-radicals derivatives of oxygen such as hydrogen peroxide. Apart from ROS, the term reactive nitrogen species (RNS) also exists, referring to nitrogen centered radicals (Halliwell & Gutteridge,

2007). ROS arise from numerous endogenous and exogenous sources (Ray et al., 2001).

*Endogenous sources of ROS:* In vivo, most ROS are generated during aerobic respiration as by-products of incomplete reduction of oxygen in mitochondria electron transport chain. ROS are also produced during the metabolism of xenobiotic compounds, the stimulation of phagocytosis by pathogens and during numerous enzymatic reactions (Nicholls & Budd, 2000).

*Exogenous sources of ROS:* ROS are also produced from a variety of lifestyle factors such as toxin and pollution exposure, radiation and electromagnetic fields, cigarette smoke, medication, anxiety, excessive exercise, dietary restrictions and saturated fats and various diseases (Cadenas & Davies, 2000). The most common ROS and RNS are:

- Superoxide anion ( $O_2^{\cdot-}$ ): is a main negatively charged free radical, which is generated as an intermediate during biochemical reactions (Halliwell, 1995), and is relatively impermeable to cell membranes. However, when superoxide is being protonated, it is converted to hydroperoxyl radical ( $HO_2^{\cdot}$ ) which can permeate cell membranes (Salvador, Sousa, & Pinto, 2001). Moreover superoxide can be produced by inflammatory cells in order to protect against invading pathological organisms (Fuchs, 1992).
- Hydroxyl radical ( $OH^{\cdot}$ ) is highly reactive and is regarded as the most harmful free radical in the biological material. It is short-lived and non cell membrane permeable (Halliwell, 1995).
- Hydrogen peroxide ( $H_2O_2$ ) is a stable and non-radical ROS that can generate free radicals such as hydroxyl radical ( $OH^{\cdot}$ ) through specific reactions (Fenton reaction). Hydrogen peroxide is permeable to cell membranes, is not capable of oxidizing lipids and proteins directly but can inactivate several enzymes (Halliwell & Gutteridge, 2007).
- Singlet oxygen ( $O_2^1$ ) is not a radical, as it does not contain unpaired electron but is highly reactive, short-lived and permeable to cell membranes (Halliwell & Gutteridge, 2007).
- Nitric oxide (NO) is a free radical and one of the numerous oxides of nitrogen. When exposed to oxygen, NO is converted into nitrogen dioxide which in turn reacts with superoxide to produce peroxynitrite (Halliwell, 1994).

- Peroxynitrite (ONOO<sup>-</sup>) is not a radical but is highly reactive. Despite its short half-life, peroxynitrite can permeate cell membranes and thus constitute a strong damaging agent of molecules, including proteins, DNA and thiol groups (Marla, Lee, & Groves, 1997).

ROS in relatively low levels play a key role in the regulation of several biological and physiological processes including the regulation of cell signaling pathways, the control of gene expression (Droge, 2002; Finkel, 2011) as well as the modulation of skeletal muscle force production (Reid, 2001b, 2008) and catalytic oxidation of some endogenous compounds and xenobiotics. In high levels, ROS can damage cellular components and molecules such as proteins, lipids and DNA by altering its structure and function (J. M. Roberts & Hubel, 2004), thus the maintenance of cellular redox balance is of high importance. For this reason cells contain a variety of antioxidant defense mechanisms to provide protection against the damaging role of the excessive ROS production and activity.

In 1985, Sies and Cadenas introduced the term “oxidative stress” as a “disturbance in the pro-oxidant and antioxidant balance in favor of the former (Sies & Cadenas, 1985). As it was referred above, cells contain antioxidant defense mechanisms which consist of enzymatic and non-enzymatic antioxidants working synergistically to scavenge ROS. The major enzymes that convert ROS to less reactive molecules are superoxide dismutase, catalase and glutathione peroxidase (Zhu, Zhang, Amin, & Li, 2008).

*Superoxide dismutase (SOD)*: SOD dismutates superoxide radicals to form hydrogen peroxide and oxygen. There are three isoforms of superoxide, one of them is located in the extracellular space and the other two are found in the intracellular space, cytosol and mitochondria (Suzuki et al., 2000).

*Catalase (CAT)*: Catalase is a homotetrameric heme-containing enzyme which catalyzes the breakdown of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub> (Kirkman & Gaetani, 2007). It is located in peroxisomes but in some cell types it can also be found in cytosol and nucleus (Legakis et al., 2002; Yamamoto, Volk, & Fahimi, 1992).

*Glutathione peroxidase (GPx)*: Glutathione peroxidase catalyzes the reduction of H<sub>2</sub>O<sub>2</sub> or hydroperoxide (ROOH) to H<sub>2</sub>O and alcohol (ROH), using reduced glutathione (GSH) (Bjornstedt, Kumar, Bjorkhem, Spyrou, & Holmgren, 1997). Three

isoforms of GPx exist and are localized in cytosol and mitochondria (Brigelius-Flohe, 1999).

Some of the main non-enzymatic antioxidants are glutathione (GSH), uric acid, bilirubin, and coenzyme Q10.

*Reduced Glutathione (GSH):* Glutathione is the most abundant non-protein thiol which is synthesized in liver and transported to tissues via the bloodstream (Meister & Anderson, 1983). GSH acts as an ROS scavenger donating its hydrogen atom (Yu, 1994) and also act as a substrate, for GPx to reduce H<sub>2</sub>O<sub>2</sub> or hydroperoxide, through its conversion to oxidized form GSSG. The enzyme glutathione reductase reduces GSSG back to GSH using NADPH as an electron donor. The ratio of reduced glutathione to oxidized glutathione (GSSH/GSSG) is used for the evaluation of cellular toxicity (Meister & Anderson, 1983; Pastore et al., 2001). Moreover, another role of GSH is to maintain exogenous antioxidants like Vitamin E and C in their reduced forms (Hughes & Kilpatrick, 1964).

*Uric acid:* Uric acid is not only a by-product of purine metabolism but it can also maintain protection against oxidative damage acting as an electron donor and scavenging peroxy radicals, hydroxyl radicals and singlet oxygen (Howell & Wyngaarden, 1960; Sevanian, Davies, & Hochstein, 1985).

*Bilirubin:* Bilirubin is known as the end product of hemoprotein catabolism but also, is considered to protect cells against harmful peroxy radicals and hydrogen peroxide (Baranano, Rao, Ferris, & Snyder, 2002; Stocker, Yamamoto, McDonagh, Glazer, & Ames, 1987).

*Coenzyme Q<sub>10</sub>:* Coenzyme Q<sub>10</sub> is primarily known as a component of mitochondria electron transport which participates in aerobic cellular respiration and ATP production (Halliwell & Gutteridge, 2007). It can inhibit lipid peroxidation by preventing the production of lipid peroxy radicals (Crane, 2001).

Apart from cell's endogenous antioxidant mechanisms, the defense against ROS is incomplete without exogenous reducing compounds such as Vitamin E, Vitamin C, carotenoids and polyphenols with the diet to be their main source (Ratnam, Ankola, Bhardwaj, Sahana, & Kumar, 2006) (André, Larondelleb, & Evers, 2010; Biehler &

Bohn, 2010). Endogenous and exogenous antioxidants act synergistically to maintain or to re-establish cell's redox homeostasis.

Except for measuring oxidants and antioxidant molecules, evaluating oxidative stress involves also the quantification of oxidatively modified molecules. Protein carbonyls (PC) is a biomarker which is indicative of protein oxidation, evaluation of malondialdehyde (MDA) and isoprostanes biomarkers are characteristics of lipid peroxidation and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) levels are indicative of DNA oxidation (M. J. Davies, Fu, Wang, & Dean, 1999; E. S. Hwang & Kim, 2007; Kasai, 1997; Lawson, Rokach, & FitzGerald, 1999).

### **3.2 The role of Oxidative stress in CKD- Blood levels**

In the literature there is strong evidence suggesting that uremia, which develops in tandem with renal deterioration, is accompanied by an enhanced state of oxidative stress, which in turn is associated with higher risk of developing cardiovascular disease (CVD) and mortality (Bayes et al., 2006). The presence of oxidative stress in uremic patients is manifested by an increase in the levels of several oxidative damage markers and by a parallel reduction in their antioxidant defense capacity (Stenvinkel & Barany, 2002; Terawaki et al., 2004). In CKD patients this state of redox imbalance is linked to additional pathological complications, such as malnutrition, anemia, and cardiovascular disease including atherosclerosis (Grune et al., 2000; Macdougall & Cooper, 2002; Stenvinkel & Barany, 2002).

CVD remains the leading cause of death in CKD patients, with oxidative stress being implicated in the onset and the development of atherosclerosis, via mechanisms such as oxidation of low-density protein (LDL) (Gosmanova & Le, 2011). It is reported that levels of lipid and protein peroxidation markers, such as malondialdehyde (MDA), advanced glycosylation end-product (AGE), and advanced oxidation protein products (AOPPs) are elevated in CKD patients, with some studies indicating a correlation between some lipid peroxidation markers and intima-media thickness (Drueke et al., 2002; Shoji et al., 2003; X. B. Yang et al., 2005). Lipid peroxidation could be described as a process where oxidants, such as free radicals, assault lipids especially in membranes (Ayala, Munoz, & Arguelles, 2014).



Additionally, regarding erythrocyte membrane integrity, modifications due to oxidation and the increased membrane rigidity could play a key role in shortening erythrocyte half-life compounding the development of anemia (Peuchant et al., 1997), which characterizes these patients. Furthermore, a disturbance of the Glutathione antioxidant system, including overall compromised blood levels of reduced glutathione (GSH), increased oxidized glutathione (GSSG), and increased erythrocyte glutathione peroxidase (GPx) and glutathione reductase (Gr-reductase) activities, has been reported in CKD (Ceballos-Picot et al., 1996; Ross, Koo, & Moberly, 1997). Likewise, a decrease in catalase (CAT), a key enzyme for the detoxification of hydrogen peroxide and organic hydroperoxide, accompanied by increased nitric oxide (NO) inactivation and protein nitration by reactive oxygen species (ROS) (Sindhu et al., 2005), have been linked to the pathogenesis of CKD related hypertension, via a dysregulation of NO function.

A variety of mechanisms have been implicated in the generation of oxidative stress in uremic patients, such as antioxidant deficiency, chronic inflammation (Hensley et al., 2000; Zimmermann et al., 1999) and, for the end-stage patients (ESRD), neutrophil activation during hemodialysis (HD) therapy (Grooteman et al., 1997), with dialysis treatment *per se* contributing in the increase of oxidative damage observed in patients with CKD (Miyazaki et al., 2000; Morena et al., 2000; Nourooz-Zadeh, 1999). Four main factors have been proposed to be responsible for the harmful effects of oxidative stress in patients under HD : the uremic milieu, the HD treatment *per se*, the hemoincompatibility of dialysis system and the concomitant drug treatment (Canaud et al., 1999).

Until today most studies examining the redox status in CKD patients focused on the end stage of the disease. However, it remains unclear when, during disease progression, the redox imbalance manifests itself, and which one of its components, defense capacity or ROS overproduction, becomes critical first.

### **3.2.1 Pre-dialysis patients and redox status**

Regarding lipid peroxidation damage, MDA levels were assessed in plasma or red blood cells (RBC) of CKD patients. Specifically, Peuchant *et al* 1996 (Peuchant et al., 1997) found increased levels of RBC free MDA in 16 patients with stage - 5 CKD

compared to age-matched controls. Additionally, Sahni *et al* (Sahni, Gupta, Rana, Prasad, & Bhalla, 2012) found increased RBC MDA levels in 60 patients with severe (near stage 5) and in 85 patients with moderate CKD compared to age-matched controls, with RBC MDA levels being significantly higher in the severe compared to moderate disease group. MDA levels were also evaluated in plasma and Papavasiliou *et al* (Papavasiliou, Gouva, Siamopoulos, & Tselepis, 2005) found them increased in 23 patients with stage 3 - 5 CKD, compared to age-matched controls. In the same study 13 patients on stages 1 - 2 CKD, exhibited a tendency for higher MDA levels compared to healthy controls but values' differences did not reach statistical significance. In contrast, stages 1 - 2 patients, exhibited significantly lower MDA levels compared to the stages 3-5 CKD patients (Papavasiliou *et al.*, 2005). Bober *et al* 2010 (Bober *et al.*, 2010), measuring TBARS (which are expressed as equivalents of MDA levels) reported higher plasma levels in 21 pre-dialysis CKD patients patients on conservative treatment compared to age-matched healthy controls. However it was not clear if results were statistically significant, nor was there information on the pre-dialysis stage of those patients. Choi *et al* (Choi *et al.*, 2011) reported that xanthine oxidase (XO) activity was increased in the plasma of 16 patients with stage - 4 CKD (almost ~1.5 fold compared to values of healthy individuals), indicating an increase in free radicals generation.

The results of these five studies concur to that CKD progression leads gradually to a state of increased ROS production, which is reflected by the elevated levels in markers of lipid damage and the decrease in the concentration of antioxidant molecules and activity of antioxidant enzymes. As mentioned already, lipid peroxidation affects erythrocyte half-life, as its excess could overcome cell repair capacity (Ayala *et al.*, 2014). According to Peuchant *et al* (Peuchant *et al.*, 1997) their results could be attributed to the fact that patients with CKD have decreased erythrocytes survival. Thus erythropoiesis is activated and could increase RBC precursors and enzymatic synthesis as well as consumption of antioxidant enzymes. Moreover, additional mechanisms are implicated in the enhanced lipid peroxidation in CKD such as the reduction in glucose-6-phosphate dehydrogenase activity, which

**Table 3.1.** Redox status in pre-dialysis CKD patients.

Authors	Groups	MDA	XO	GSH	SOD	CAT	GPx	
(Peuchant et al., 1997)	a. 16 CKD patients, (age: 43.6±11.2 yr) GFR:12.6±5.4 ml/min b. 26 healthy subjects, (age: 43.6±11.2 yr)	RBC free MDA: a.↑ 3.88±2.67 b. 1.74±0.56 Total MDA: a.10.0±9.31 b.7.85±2.82 (nmol/ml)				a.800 b.790 (U/g Hb)	a.45 b.43 (U/g Hb)	a.34 b.32 (U/g Hb)
(Papavasilou et al., 2005)	a.12 CKD patients, (age: 66 yr) Creatinine clearance:23.9±6.6 ml/min b. 11 CKD patients (age: 61 yr) Creatinine clearance:20.2±6.5 ml/min c. 13 CKD patients (age: 63 yr) Creatinine clearance:87.2±7.1 ml/min d. 15 healthy subjects (age: 59 yr)	Plasma: a. ↑1.2 b.↑1.25 c.1.15 †† d. 0.9 (nmol/ml)					a.↑ 15.50±5 b.↑ 15.20±4.70 c.↑ 11.30±2.88 d. 9.98±3.33 (IU/g Hb)	
(Bober et al., 2010)	a.21 CKD patients (age: 56.8±16.0 yr) b. 21 healthy subjects (age: 56±16.6 yr)	Plasma: a.1.05±0.21 b.0.79±0.12 (μmol/L)		a.↑8.26±1.16 b. 7.26±1.50 (nmol/g Hb)				
(Choi et al., 2011)	a.16 CKD patients GFR:23.56±10.64 ml/min/1.73 m <sup>2</sup> b. 18 healthy subjects (age: 48.9±15.4 yr)		a.↑6.3±1.5 b. 4.5±0.9 (RLU)					
(Sahni et al., 2012)	a. 60 CKD patients, (age: 43.05±11.9 yr) GFR:12.75±5.52 ml/min b. 85 CKD patients, (age: 42.88±11.4 yr) GFR:28.61±12.93 ml/min c. 40 Healthy subjects (age: 42.42±2.2 yr)	RBC : a.↑4.89±1.33 † b.↑2.77±1.015 c. 1.13±0.219 (μmol/g Hb)		a.↓0.61±0.40 † b.↓1.05±0.334 c.1.87±0.608 (μmol/g Hb)	a.↓994.57±87.14 † b.↓1136.99±101.56 c.1382.55±93.93 (IU)	a.↓125.86±17.36 † b.↓139.19±24.35 c. 184.66±17.29 μL/min/ml Hb)		

MDA: malondialdehyde; XO: xanthine oxidase; GSH: reduced glutathione; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; yr: years; † versus healthy controls, † difference between a and b groups, †† difference between a and b groups with c group.

leads to a decrease in NADPH and GSH concentrations and vitamin E deficiency (Schiavon, Guidi, Biasioli, De Fanti, & Targa, 1994; Yalcin et al., 1989; Yawata & Jacob, 1975).

Undeniably, a compromised antioxidant intake may contribute to the above observations, as pre-dialysis patients need to adhere to specific diet guidelines and restrictions (Sahni et al., 2012). Indeed, intake of exogenous antioxidant vitamins, such as vitamins A and C, and glutathione precursors, were found to be at lower levels in pre-dialysis patients compared to healthy subjects, with nutritional intakes worsening as CKD became more severe, and evident negative correlations between antioxidant intakes with oxidative stress levels (Sahni et al., 2012), (Table 3.1).

### **3.2.2 Interventions in pre-dialysis patients and redox status**

At the pre-dialysis stage, several interventions to correct for anemia, counteract inflammation and supplement nutrition have become part of clinical and peri-clinical care in addition to appropriate pharmacological therapy, and could affect redox balance.

Ganguli *et al* (Ganguli et al., 2009), reported that various forms of intravenous iron [low molecular weight iron dextran (ID), sodium ferrigluconate complex, in sucrose (SFGC), and iron sucrose (IS)] at clinically used doses, resulted in elevated MDA levels, measured immediately after the iron transfusion period (Ganguli et al., 2009). Marsillach *et al* (Marsillach et al., 2007) evaluated the 6-months effects of EPO treatment, together with iron treatment (SFGC or IS), measuring antibodies against ox-LDL, and paraoxonase (PON1) activity and concentration which is assumed to play an important role in oxidized lipid degradation. Results revealed decreased levels of ox-LDL antibodies and increased serum PON1 activity despite the iron treatment (Marsillach et al., 2007). However, in addition to an effect of EPO, large differences in baseline LDL and ferritin levels between the two studied cohorts can explain the conflicting reports.

Ramos *et al* (L. F. Ramos et al., 2011) investigated whether the administration of mixed tocopherols and lipoic acid for 2 months could modify oxidative stress indices in CKD patients. However, no significant changes in F<sub>2</sub>- isoprostanes and protein thiols concentrations were observed in these patients compared to matching

controls. As the authors discussed, the sample size, the intervention's duration, the dose or the composition of antioxidants all could probably have been biologically ineffective in altering the redox status of this particular patient population (L. F. Ramos et al., 2011). Moreillon *et al* (Moreillon et al., 2013) assessed the antioxidant effects of two types of herbal supplements curcumin and boswellia serrata, on GPx levels, after 8 weeks supplementation. Despite the increase in plasma GPx levels in the treatment group, results were not statistically significant. Perhaps the duration of supplementation was not sufficient enough for the small sample size used (Moreillon et al., 2013) to detect an effect.

Data in non-CKD patients suggest that higher doses of vitamin E supplementation may be necessary to observe measurable changes in redox status. For example, in hypercholesterolemia patients, after 4 months of Vitamin E supplementation, there was a decrease in F2-isoprostane concentration, only with the high doses of 1600 and 3200 IU/day (L. J. Roberts, 2nd et al., 2007). Herbal supplements such as curcumin (Hatcher, Planalp, Cho, Torti, & Torti, 2008) and Boswellia serrata, (Gupta et al., 2001) (Madisch et al., 2007), are known for their anti-inflammatory and antioxidant characteristics, for example in patients with colitis (Gupta et al., 2001) (Madisch et al., 2007). While one study (Moreillon et al., 2013) did not report an effect, future studies could explore further their possible contribution to ameliorate oxidative stress in pre-dialysis patients via nutritional strategies.

Apart from the impaired nutritional status, CKD patients are also characterized by iron deficiency and depletion of iron storage. Although the administration of intravenous iron is fully recommended in these patients for anemia correction, the short and long-term safety of this administration remains unclear. According to Bishu and Agarwal (Bishu & Agarwal, 2006) intravenous iron administration contributes to elevated oxidative stress and endothelial dysfunction in CKD patients. Moreover, according to Himmelfarb *et al* (Himmelfarb, Stenvinkel, Ikizler, & Hakim, 2002) endothelial dysfunction plays a key role in the pathogenesis of atherosclerosis in CKD patients and oxidative stress is implicated in this pathway. It can be surmised that a treatment meant to correct anemia, iron supplementation, could exacerbate atherosclerosis by promoting oxidative stress. The two studies supplementing iron to pre-dialysis patients reviewed above, Ganguli *et al* (Ganguli et al., 2009), and Marsillach *et al* (Marsillach et al., 2007) reported conflicting effects of iron on lipid peroxidation. However the interaction of iron to circulating LDL cannot be

underestimated (i.e. a systemically atherogenic environment) while at the same time EPO appears to confer an antioxidant advantage (as shown earlier by Papavasileiou *et al* (Papavasiliou et al., 2005), that EPO treatment prevented the reduction in erythrocytes GPx activity observed in patients not receiving EPO).

### 3.2.3 HD patients and redox status

According to literature, increased levels of MDA (Bober et al., 2010; Dimitrijevic et al., 2012; Guo, Chen, Hsu, & Wang, 2013; Haklar, Yegenaga, & Yalcin, 1995; Sakata et al., 2008; Sommerburg et al., 1998; Triolo et al., 2003) were observed in HD patients compared to healthy individuals, reflecting extensive lipid damage. Sommerburg *et al* (Sommerburg et al., 1998), reported that HD patients who received long-term EPO treatment showed decreased levels of lipid peroxidation compared to patients who did not receive EPO treatment. Furthermore, Dolegowska *et al* (Dolegowska et al., 2007) reported a significant increase in plasma isoprostanes (8-iPF<sub>2</sub>a-III) concentrations, in HD patients.

Regarding protein damage, levels of protein carbonyls were also found to be elevated in HD patients in four studies (Anraku et al., 2004; Haklar et al., 1995; Mera et al., 2005; Ward, Ouseph, & McLeish, 2003). In addition, advanced oxidation protein products (AOPPs) were found to be in high levels in HD patients in two studies (Sakata et al., 2008; Ward et al., 2003) however they were reported to be no different than controls in one study (Fragedaki et al., 2005). In the study of Fragedaki *et al* (Fragedaki et al., 2005) patients were on dialysis for a shorter period (an average of 3 years) than the other studies (Sakata et al., 2008; Ward et al., 2003) a fact that could affect AOPPs levels. Finally, Choi *et al* (Choi et al., 2011) reported increased XO activity in HD patients compared to healthy subjects (by almost 3 fold); with XO activity being also higher (by 2.1 fold) than pre-dialysis patients. Taking into consideration the above results, there is evidence of extensive oxidative damage to total protein content and lipids in HD patients.

Regarding the antioxidant capacity in HD patients, the literature provides differing results. Sakata *et al* reported a significant increase in 'global' antioxidant capacity markers, such as ORAC and TEAC (Sakata et al., 2008). On the other hand, Dimitrijevic *et al* (Dimitrijevic et al., 2012) reported a significant decrease in total

antioxidant capacity (TAC) of HD patients compared to controls while decreased albumin levels were also observed. It should be noted that these global antioxidant capacity markers do not represent innate antioxidant capacity but rather represent global availability of free radical scavenging compounds, whose levels are greatly affected by nutrition.

Moreover, the activity of the free radical scavenging enzymes plasma GPx (GSH-Px) and SOD was found to be significantly decreased in HD patients (Guo et al., 2013). GPx has been identified in two forms: cellular or cytosolic (GPx-1) which is presented in red blood cells and the cytosol of almost all tissues, and extracellular (GPx-3) in the plasma, with selenium (Se) being a basic structural component in both forms, (Guo et al., 2013), an element also affected by nutrition. Additionally, Gr Reductase activity was found to be significantly higher in HD patients compared to healthy subjects (Stepniewska et al., 2006), probably reflecting the organism's efforts to activate the antioxidant defense. Likewise, that study also reported increased GSH total blood concentrations in the HD patients studied (Stepniewska et al., 2006). On the other hand, two studies (Dimitrijevic et al., 2012),(Ward et al., 2003) reported a large reduction of free sulfhydryl groups levels in HD patients, measured in plasma.

All the results (Table 3.2) concur to that HD treatment seems to contribute to augmented oxidative stress. Most of the studies observed increased levels of protein and lipid damage in HD patients, which were measured with established biomarkers. As it appears, HD treatment causes an enhanced rate of LDL oxidation, which leads to the development of atherosclerosis, as reflected in elevated blood MDA levels. One mechanism is that the uremic milieu and the HD process *per se* stimulate neutrophils, a procedure known as "neutrophil burst", which directly or indirectly results to hydrogen peroxide production. This is then converted to hypochlorous acid, which in turn contributes to the oxidation of plasma proteins and LDL, assaulting their membranes (Morena, Delbosc, Dupuy, Canaud, & Cristol, 2005; Sakata et al., 2008). Furthermore, as it was referred above, lipid peroxidation negatively affects erythrocyte half-life leading to decreased survival and manifestation of anemia. EPO appears to help as Sommerburg *et al* (Sommerburg et al., 1998) reported that HD patients who received long-term EPO treatment presented decreased levels of lipid peroxidation. These results in HD concur with findings in pre-dialysis patients, where also EPO appears to confer an antioxidant advantage (Papavasiliou et al., 2005), by preventing the reduction in erythrocytes GPx activity observed in patients not

**Table 3.2. Redox status in HD patients**

Authors	Groups	HD modality & period	TBARS /MDA	Free sulfhydryl groups	Isoprostanes8-iPF2a-III	AOPP	Protein Carbonyls	XO	GSH	ORAC/ TEAC/ TAC	SOD/ GPx/ GR Reductase
(Haklar et al., 1995)	a. 14 HD patients (age: 52 yr) b. 14 healthy subjects (age: 40 yr)	Cuprophan, dialyzers,22 months, 3x/week/ 4 h	MDA(nmol/ml) a.↑5.50±0.6 b.2.82±0.36				a.↑41.2±10.4 b.22.6±5.5 (nmol/mg protein)				
(Sommerburg et al., 1998)	a. 8 HD patients Hb<10 g/dl, (age: 58 yr) b. 8 HD patients Hb>10 g/dl, (age: 62 yr) c. 27 HD patients receiving rHuEpo (age: 66 yr) d. 20 healthy subjects (age: 59 yr)	Bicarbonate HD, 6-10 years,3x/week/4-5 h	MDA (µM) a.↑3.81±0.86 b. ↑ 2.77±0.58 c. ↑ 2.50±0.12 d. 0.37±0.03								
(Ward et al., 2003)	a. 11 HD patients (age: 51± 5 yr) b. 12-17 healthy subjects (age 23-54 yr)	49±11 months		a.↓ 268±22 b.438±16 (µmol/ L)		a.↑191±27 b. 74±8 (µmol/ L)	a.↑0.144± 0.037 b. 0.041± 0.008 (nmol/mg)				
(Choi et al., 2011)	a.18 patients, age 52.0±17 yr, b.16 healthy subjects (age: 48.9±15.4 yr)	94.3±43.5 months, 3x/wk/4h						a.↑ 13±9.4 b. 4.5±0.9 (RLU)			
(Triolo et al., 2003)	a. 10 HD patients (age: 64.6±13.7 yr) b. 30 healthy subjects (age: 59.8±9.4 yr)	Bicarbonate HD, 86.6±47.2 months, 3 x/week/3.5-4 h	MDA ( nmol/mL) a.↑1.87±0.36 b. 1.13±0.18								
(Anraku et al., 2004)	a. 11 HD patients (age 25 to 87 yr) b.11 age-matched healthy subjects	Bicarbonate,1- 9 years, 3 x/week/4-5 h					a.↑ 1.0±0.1 b.0.4±0.03 (nmol/mg protein)				
(Fragedaki et al., 2005)	a. 12 SHD patients (age: 57.5±12.8 yr) b. MHD 13 patients (age: 48±11.5 yr) c. 12 healthy subjects (age: 52.9±10.7 yr)	a.Polyethersulfone, 3.6±1.82 years, 3 x/week/4-4.5 h b.Polyethersulfone, 3.3±1.37 years, 6 x/week/2-2.5 h				a.0.44±0.23 b.0.57±0.36 c. 0.60±0.46 (nmol/mg protein)					



Authors	Groups	HD modality & period	TBARS /MDA	Free sulfhydryl groups	Isoprostanes8-iPF2a-III	AOPP	Protein Carbonyls	XO	GSH	ORAC/ TEAC/ TAC	SOD/ GPx/ GR Reductase
Mera et al., 2005)	a. 20 HD patients (age 62.8±12.7 yr) b. 10 healthy subjects (age 67.8±1.8 yr)	Bicarbonate HD,1-9 years, 3 x/week/4-5 h					a.↑3.12±1.1 b.2.10±0.34 (nmol/mg protein)				
(Stepniewska et al., 2006)	a. 25 HD patients (age 50.3±13.79 yr) b. 26 HD patients (age 60.54±13.54 yr) c.29 healthy subjects (age 49.7±11.4 yr)	a. Polysulfone HD, glucose-free fluid, 27.44±15.87 months,3 x/week,4h b. Polysulfone HD, glucose fluid, 24.43±12.68 months 3 x/week/4h							a.↑19.6±8.8 b.↑20.5±8.0 c. 14.2±2.1 (μmol/g Hb)		Gr Reductase a.↑2.82±0.98 b.↑2.57±0.76 c. 1.95±0.40 (U/g Hb)
(Dolegowska et al., 2007)	a.22 HD patients (age: 53.06±11.43 yr) b.22 HD patients (age: 57.70±14.78 yr) c. 22 healthy subjects (age: 51.93±9.94 yr)	a. Polysulfone HD. glucose-free fluid, 3 x/week/4h b. Polysulfone HD, glucose fluid,3 x/week/4h			a.Plasma: 0.05±0.02 RBC: 7.19±10.45 b.↑Plasma:0.19±0.15 RBC:8.75±7.58 c.Plasma:0.10±0.05 RBC:5.29±7.54 (ng/ml), (ng/g Hb)						
(Sakata et al., 2008)	a.36 HD patients (age: 63.6±12.1 yr) b.15 healthy subjects (age: 32.5±8.6 yr)	Bicarbonate HD, 12.1±7.0 years	MDA (μM) a.↑0.25±0.11 b.0.05±0.02				a.↑88.8±39.7 b.43.8±20.3 (μM)			a.ORAC:↑ 2,672±554 TEAC:↑ 0.77±0.2 b.ORAC: 1,363±174 TEAC: 0.43±0.12 (U/ml), mM Trol eq/l	
(Bober et al., 2010)	a.22 HD patients (age: 55.9±14.8 yr) b.23 HD patients (age: 64.3±12.1 yr) c. 21 healthy subjects (age: 56±16.6 yr)	a. Polysulfone HD, glucose-free fluid, 8.78±6.42 months, 3 x/week b. Polysulfone HD glucose fluid, 9.45±6.62 months 3x/week	MDA a.↑1.56±0.27 b.↑1.36±0.30 c.0.79±0.12 (μmol/L)								

Authors	Groups	HD modality & period	TBARS /MDA	Free sulfhydryl groups	Isoprostanes8-iPF2a-III	AOPP	Protein Carbonyls	XO	GSH	ORAC/ TEAC/ TAC	SOD/ GPx/ GR Reductase
(Dimitrijevic et al., 2012)	a.15 HD patients (age: 55.6±18.2 yr) b.29 healthy subjects (age: 55±15.8 yr)	Bicarbonate HD 52.5±61.6 months	MDA a.Plasma: ↑11.3±11.2 RBC: ↑14.7±2.2 b.Plasma: 6.0±1.1 RBC: 8.7±1.3 (mmol/l),(nmol/ml)	a. ↓284.4±44.5 b.449.2±66.6 (μmol/ L)						a.TAC: ↓ 2.5±0.3 b. TAC: 3.6±0.5 (μmol/ L)	
(Guo et al., 2013)	a.20 HD patients (age:55±7 yr) b.25 healthy subjects (age: 53±7 yr)	HD 6±1 years, 3 x/week/4 h	MDA a. ↑6 (median) b. 2.9 (nmol/L)								a. GPx: ↓ 50.5±8.4 SOD: ↓ 3.4 b. GPx: 85.2±6.1 SOD: 8.3 (U/ml) (median)

TBARS: Thiobarbituric acid reactive substances; MDA: malondialdehyde; AOPP: Advanced Oxidation Protein Products; GSH: reduced glutathione; ORAC: Oxygen Radical Absorbance Capacity; TEAC: Trolox Equivalent Antioxidant Capacity; TAC: Total Antioxidant Capacity; SOD: superoxide dismutase; GPX: glutathione peroxidase; yr: years; ↑ versus healthy controls

receiving EPO. Moreover, while erythropoiesis is, in a healthy individual, stimulated by ROS, as discussed by Migliaccio, erythropoiesis also counteracts circulating ROS levels via increases in available catalase and possibly other antioxidant enzymes (Migliaccio, 2013). Further on the atherosclerosis mechanism, the presence of F<sub>2</sub> – isoprostanes has been determined in human atherosclerotic lesions (Pratico et al., 1997). F<sub>2</sub>–isoprostanes are created during the non-enzymatic peroxidation of arachidonic acid bound up with phospholipids of cells membranes and lipoproteins (Cracowski et al., 2001; Morrow et al., 1990; L. J. Roberts, 2nd & Morrow, 2002), and they are considered sensitive and specific indicators of oxidative stress intensity in vivo (Dillon, Lowe, Billington, & Rahman, 2002; Pratico et al., 1997). Given reports, such as (Dolegowska et al., 2007), of a significant increase in plasma isoprostanes in HD patients, the link between oxidative stress and CVD in HD patients is further highlighted.

The results on the antioxidant capacity of HD patients are not as straightforward to explain. Caloric restriction (without essential nutrient deficiency) in rats has been shown to result in decreased antioxidant capacity (ORAC) (Cao, Prior, Cutler, & Yu, 1997). CKD patients however, are subject to both caloric restriction and deficient antioxidant intakes [23]. Differences between studies reporting on HD antioxidant capacity could be thus attributed not only to adaptive mechanisms, due to years in dialysis and comorbidities, but also to dietary restrictions and inter-individual differences in antioxidant nutritional intakes, as already reported in pre-dialysis patients (Sahni et al., 2012).

Additionally, the hemodialysis process *per se* may compromise key nutrient and trace mineral levels as highlighted with regards to levels of Se, a basic component of GPx. Guo *et al* (Guo et al., 2013) reported that HD patients presented with lower plasma Se concentrations than healthy subjects and this could be attributed to either impaired diet/absorption or increased loss of Se during dialysis treatment (Pakfetrat, Malekmakan, & Hasheminasab, 2010). Additionally, taking into account that the kidney is the main site of plasma GPx synthesis and is also capable of Se accumulation (Avissar et al., 1994), that the levels of both Se and GPx are reduced in ESRD patients is not unexpected. A similar explanation could be also given for the significant reduction in plasma SOD activity observed in HD patients, linked to Zn availability, which is a structural element of SOD and whose levels tend also to be reduced in renal insufficiency (Zumkley, Bertram, Lison, Knoll, & Losse, 1979).

Regarding GSH depletion in HD patients, many mechanisms have been proposed. According to Yawata *et al.* (Yawata & Jacob, 1975), an obstruction to the pentose phosphate pathway leading to impaired production of NADPH occurs in patients with ESRD. As a result, Gr Reductase cannot recycle GSSG back to GSH using NADPH as electron source (Yawata & Jacob, 1975). Notwithstanding, in many studies, GR activity has been found to be increased or in normal levels (Ceballos-Picot *et al.*, 1996; Romeu *et al.*, 2010; Schettler, Wieland, Methe, Schuff-Werner, & Muller, 1998; Stepniewska *et al.*, 2006). Thus, GSH depletion could be alternatively attributed either to a diminished GSH synthesis and/or to an increased GSH degradation, since its precursors cystine, glutamate and glycine could be normal or elevated in patients' blood (Ceballos *et al.*, 1990).

#### **3.2.4 Redox status before and after the HD treatment**

The hemodialysis treatment can affect patients' redox status, and several studies examined the possible contribution of HD treatment on redox imbalance (Table 3.3). Three studies indicated that HD treatment *per se* augments lipid peroxidation, assessed by MDA (Bober *et al.*, 2010; Ogunro, Olujombo, Ajala, & Oshodi, 2014) and F<sub>2</sub> – isoprostanes levels (Dolegowska *et al.*, 2007) and induces protein damage, assessed by protein carbonylation (Ward *et al.*, 2003). However, there were studies, which did not observe any significant changes in lipid peroxidation (unchanged MDA levels) (R. Ramos & Martinez-Castelao, 2008; Trimarchi *et al.*, 2003), F<sub>2</sub>-isoprostanes levels (Smith *et al.*, 2003) and ox-LDL levels (Malindretos *et al.*, 2007) following the HD process (Malindretos *et al.*, 2007; R. Ramos & Martinez-Castelao, 2008; Smith *et al.*, 2003; Trimarchi *et al.*, 2003). Moreover, in the Malindretos *et al.* study, oxidized LDL levels didn't significantly change after HD treatment, neither intravenous iron administration during HD changed its concentration following HD (Malindretos *et al.*, 2007).

Regarding antioxidant capacity after completion of HD therapy, there are conflicting findings. Two studies reported that the levels of GSH and the activities of GPx and Gr-reductase increased after HD therapy (Stepniewska *et al.*, 2006; Westhuyzen, Saltissi, & Stanbury, 2003). However, there were three studies in younger patients, which observed reduced levels of GSH (Bober *et al.*, 2010; Ogunro

et al., 2014), reduced GPx activity (Ogunro et al., 2014) and reduced TAS levels (K. C. Huang et al., 2006; Ogunro et al., 2014) following HD.

Ogunro *et al* (Ogunro et al., 2014), evaluated the effects of cellulose and polysulfone membranes, which are the most commonly used types, on redox status in chronic HD patients. SOD activity was significantly reduced in both cellulose and polysulfone membrane dialysis. Regarding other antioxidant biomarkers evaluated in this study, both types of membranes caused changes to same direction but not significant in all cases. Specifically, CAT activity was significantly increased only in polysulfone membrane while GPx activity was reduced in both types but not significantly. Additionally, TAS and GSH levels were significantly reduced after cellulose membrane HD while the reductions were not significant in polysulfone membrane users. Lastly, following HD treatment, MDA levels were increased in both types but the change was significant only in cellulose membrane.

On the other hand, Ward *et al* (Ward et al., 2003), did not find differing responses to the use of polysulfone or cellulose membranes on protein oxidation. These HD patients demonstrated already high plasma protein carbonyls and AOPP concentrations compared to normal values, before HD treatment. With HD, using either membrane, protein carbonyls increased significantly over the course of HD and remained significantly elevated for the post-dialysis period, indicating a worsening in protein oxidative damage. Dialysis had no effect on AOPP levels and their values remained significantly higher than normal after dialysis. Perhaps counterintuitively, in that study, following HD a significant increase in plasma free sulfhydryl groups concentrations was found (levels essentially corrected to the levels of normal values), for both membranes used (Ward et al., 2003).

Bober *et al* (Bober et al., 2010), assessed the effects of glucose content in the dialyzing fluid on RBC antioxidant capacity. They found that glucose concentrations of about 5.6 mmol/l resulted in an augmentation of the hexose monophosphate cycle (HMP) in erythrocytes and accordingly benefited the antioxidant system. Furthermore, the group received the glucose-free HD treatment presented with increased TBARS concentration after the treatment (Bober et al., 2010). According to the authors, this probably occurred, because the free radicals produced during the glucose-free HD session could not be neutralized through the non-enzymatic pathway at a satisfactory rate (Bober et al., 2010). In the cross-sectional study of Dolegowska

*et al* (Dolegowska et al., 2007), there were no significant differences, either pre or after HD treatment, in 8-iPF<sub>2</sub>a-III levels in RBC membranes of HD patients treated.

**Table 3.3** Redox status before and after the HD treatment

Authors	Groups	HD modality & period	AOPP	Ox-LDL	Free sulfhydryl	Protein Carbonyls	TBARS /MDA	F2-Isoprostanes	GSH	SOD	GPx	GR-Reductase	CAT	TAS
(Ward et al., 2003)	11 HD patients, (age 51±5 yr)	a. polysulfone membrane b. cellulose membrane 49±11 months	a.190±37 b.163±26 (μmol/L)		a.↑425±15 b.↑408±23 (μmol/L)	a.↑0.175±0.03 b.↑0.178±0.04 (nmol/mg)								
(Westhuyzen et al., 2003)	13 HD patients, (age 69.2±15.6 yr)	Vitamin E membrane, 3x/wk a. Baseline b. at 6 wk c. at 13 wk							a.0.99±0.17 b.1.08±0.16 c.1.04±0.20 (mmol/L)	a.725±102 b.746±130 c.771±151 (U/g Hb)	a.46.8±14.8 b.↑55.7±14.8 c.↑56.7±17.2 (U/g Hb)			
(Trimarchi et al., 2003)	12 HD patients, (age 65.5±13.1 yr)	Cellulose membrane, for 20.83 months, 3x/wk					4.62±0.9 (μmol/L)							
(Smith et al., 2003)	11 HD patients, (age 64±4 yr)	Polysulfone membrane,3x/wk						602±105 (pg/ml)						
(Stepniewska et al., 2006)	a. 25 HD patients, (age 50.3±13.7 yr) b. 26 HD patients (age 60.5±13.5 yr)	a. Polysulfone membrane, glucose-free fluid, for 27.44±15.87 months,3x/week/4h b. Polysulfone glucose fluid,for 24.43±12.68 months 3 x/week/ 4h							a.↑18.3±7.2 b.19.8±8.2 (mmol/gHb)			a.2.69±0.95 b.↑2.05±0.59 (U/g H)		
(K. C. Huang et al., 2006)	25 HD patients, (age 58±3yr)	HD for 12 months												↓0.5 (mmol/l)
(Malindretos et al., 2007)	20 HD patients, (age 64.7±17.3 yr):	Polysulfone membrane, 3x/wk/ 4h		14.52±8.3 (mU/L)										

Authors	Groups	HD modality & period	AOPP	Ox-LDL	Free sulfhydryl	Protein Carbonyls	TBARS /MDA	F2-Isoprostanes	GSH	SOD	GPx	GR-Reductase	CAT	TAS
(Dolegowska et al., 2007)	a.22 HD patients, (age 53.1±11.4 yr) b.22 HD patients, (age 57.7±14.7 yr)	a. Polysulfone membrane, glucose-free fluid, 3x/week/4h b. Polysulfone glucose fluid, 3x/week/ 4h						a.↑Plasma: 0.18±0.17 RBC:7.40±11.22 b.Plasma:0.16±0.10 RBC:7.62±7.03 (ng/ml), (ng/g Hb)						
(R. Ramos & Martinez-Castelao, 2008)	34 HD patients, (age 57±1 yr)	Cellulose membrane					0.28±0.19 (ng/g HDL)							
(Bober et al., 2010)	a.22 HD patients, (age 55.9±14.8 yr) b.23 HD patients, (age 64.3±12.1 yr)	a. Polysulfone membrane glucose-free fluid, 3x/week b. Polysulfone HD glucose fluid,3x/week					a.↑2.01±0.55 b.↓1.17±0.31 (µmol/L)		a.↓ 8.82±2.22 b.↑11.07±3.28 (nmol/g Hb)					
(Ogunro et al., 2014)	a.35 HD patients, (age 51.9±12.4 yr) b. 38 HD patients, (age 49.8±10.6 yr)	a. Cellulose membrane b. Polysulfonate membrane, 3x/wk/ 4-5 h					a.↑6.05±0.9 b. 4.71±0.7 (nmol/mL)		a.↓2.09±0.3 b.3.68±0.2 (µmol/g Hb)	a.↓378±13.2 b. ↓418±19.7 (U/g Hb)	a.22.5±3.1 b. 27.5±4.2 (U/g Hb)		a.1231±41.6 b.↑1370±39.4 (U/g Hb)	a.↓1.0±0.7 b.1.16±0.2 (mmol/L trolox)

AOPP: Advanced Oxidation Protein Products; Ox-LDL: oxidized Low Density Lipoprotein; MDA: malondialdehyde; GSH: reduced glutathione; SOD: superoxide dismutase; GPX: glutathione peroxidase; CAT: Catalase; TAS: Total Antioxidant Status; yr: years; ↑:versus before the HD treatment; ↓: versus before the HD treatment;



with glucose or glucose-free dialyzing fluids. Still, they reported a significant increase of plasma 8-iPF<sub>2</sub> -III concentration following HD in the glucose-free group (which however started at a much lower level than the glucose treated group).

Various mechanisms have been proposed to account for the increased oxidative damage following HD treatment. Firstly, the issue of hemoincompatibility induced by the contact between human blood and the non-biological materials of the hemodialyzer and the resulting effects on leukocyte and platelet activation and inflammation (Fumeron et al., 2005; Morena et al., 2000; Washio et al., 2008). Moreover, systemic inflammation effects mediated via neutrophil activity burst resulting in the release of ROS into the bloodstream (Morena et al., 2005; Sakata et al., 2008). Furthermore, the role the HD process *per se* in reducing plasma antioxidant defense because several antioxidant dialyzable solutes are removed during HD.

The role of the type of dialysis membrane used is not straightforward (Trimarchi et al., 2003; Ward et al., 2003). This is probably due to the different reactions that take place in sulfhydryl groups and protein carbonyl formation. Possibly, protein sulfhydryl groups are oxidized reversibly through small molecules oxidants, which can be removed by dialysis process, while AOPP and protein carbonyls may represent a chronic state of irreversible oxidative damage (Ward et al., 2003).

With regard to dialyzing fluid composition, it could be generally concluded that glucose has a beneficial effect on the antioxidant properties of RBC and protects them by decreasing the risk for hemolysis (Bober et al., 2010; Dolegowska et al., 2007), (Table 3).

### **3.2.5 Intervention in HD**

Three studies examined the effects of Vitamin C administration on redox status of HD patients. Fumeron *et al* (Fumeron et al., 2005) reported that oral administration of 250 mg Vitamin C three times per week for two months, could not either increase GSH or reduce protein carbonyls levels. Washio *et al* (Washio et al., 2008), found that oral administration of Vitamin C for 3 months, ranging from low (200 mg) to high dose (1000 mg) could not suppress the enhancement of Cu/Zn-SOD expression, an oxidative stress marker. On the other hand, Tarng *et al* (Tarng, Liu, & Huang, 2004)

found that intravenously administration of 300 mg Vitamin C for 8 weeks decreased significantly 8-OHdG contents in cellular DNA of lymphocytes. Moreover in the same study vitamin C significantly reduced intracellular ROS production of lymphocytes of patients on HD (Tarng et al., 2004).

Regarding Vitamin E, another important antioxidant substance, Smith *et al* (Smith et al., 2003) examined the effects of 400IU administration for 2 months, on redox status of 11 HD patients. The elevated free F2-isoprostane plasma concentrations were not decreased by vitamin E supplementation. Likewise, treatment with a combination of mixed tocopherols plus *α*lipoic acid (oral) for 6 months didn't change plasma F2- isoprostane and F2- isofurane of HD patients compared to placebo group as reported by Himmelfarb *et al* (Himmelfarb et al., 2014).

A pilot study by Trimarchi *et al* (Trimarchi et al., 2003) showed that the daily administration of 1,200 mg NAC for one month could significantly reduce plasma lipid peroxidation of HD patients compared to the control group. Finally, Fatouros *et al* (Fatouros et al., 2010) reported that daily administration of 20 mg/kg L-carnitine in 12 HD patients for 8 weeks resulted in 2,7 fold increased GSH/GSSG ratio, a 4.5% increase in glutathione peroxidase activity, a 19% decrease in MDA levels and 27% decrease in protein carbonyls concentration.

Vitamin C is one of the most important water-soluble antioxidants. There is evidence that HD patients exhibit a 30-50% decrease in Vitamin C levels after dialysis treatment, as reviewed elsewhere (Deicher & Horl, 2003). Moreover, reduced Vitamin C levels in HD patients have been associated with an increased risk for CVD morbidity and mortality (Deicher, Ziai, Bieglmayer, Schillinger, & Horl, 2005). However, elsewhere, only intravenous administration was found to have a measurable beneficial effect (Tarng et al., 2004). Thus, oral administration of Vitamin C may not be sufficient to correct its deficiency in HD.

Oral administration of Vitamin E did not decrease plasma isoprostanes in HD patients (Deicher et al., 2005; Malindretos et al., 2007). In contrast, it has been reported that long-term administration of 200 mg of Vitamin E decreased F2-isoprostanes plasma levels in mildly hypercholesterolemic men (Kaikkonen et al., 2001). Moreover in healthy subjects Vitamin E administration resulted in lower F2-isoprostane concentrations in urinary samples (H. Y. Huang et al., 2002). Perhaps differences in outcomes relate to dosage and duration of administration could explain these results, notwithstanding the augmented oxidant production characterizing HD

patients which perhaps oral administration of Vitamin E, at safe levels for these patients, could not combat.

N-acetylcysteine (NAC) is a ROS scavenger and its administration seems to increase glutathione concentration given that it raises intracellular levels of one of its precursors, cysteine (Afaq, Abidi, & Rahman, 2000; Aruoma, Halliwell, Hoey, & Butler, 1989; Vendemiale et al., 2001). Along with L-Carnitine, these products showed promising results and should be further examined in larger patient cohorts.

### 3.2.6 Other type of Dialysis and redox status

In the literature, there have been also studies that examined other types of dialysis in relation to oxidative stress and antioxidant status biomarkers (Table 3.4). A metabonomics study by Choi *et al* examined the effects of different dialysis modalities on serum profiles of patients (Choi et al., 2011). XO activity levels were similar for both HD and peritoneal dialysis (PD) patients. Still XO activity was significantly higher in PD compared to controls and to non-dialysed uremic patients (non HD or PD) (Choi et al., 2011), perhaps justifying the need to address it pharmaceutically (see below *Interventions in PD*).

In the study of Canestrari *et al* (Canestrari et al., 1995) erythrocyte GSH levels were unchanged or slightly increased in the patients compared to the control group while a nearly 3-fold increase in GSSG was observed in the PD group (Canestrari et al., 1995). In the same study, plasma GSH levels were significantly decreased in the PD group compared to control group and GSSG levels were slightly increased in PD patients (Canestrari et al., 1995). Moreover, increased RBC and plasma TBARS levels were found in PD patients compared to control group. Finally, significantly higher erythrocyte GSH-Px activity was observed in the PD group while plasma GSH-Px activity was similar in PD and controls (Canestrari et al., 1995).

Moreover, a study by Gonzales-Diez *et al* (Gonzalez-Diez et al., 2012), examined and compared the effects of haemodiafiltration (HFR) to HD with polysulfone membranes (HD-PS). The HFR group demonstrated moderate changes in oxidative stress biomarkers and antioxidant capacity markers, indicating that this method may in the long term preserve a more stable and balanced redox status compared to HD-PS (Gonzalez-Diez et al., 2012), (Table 4).

**Table 3.4.** Other modalities of HD and redox status

Authors	Groups	HD modality	TBARS/MDA	Protein Carbonyls	GSSG	GSH	SOD	GPx	XO	CAT	TAC
(Canestrari et al., 1995)	a. 18 patients (age 62.9±13.9 yr) b. 15 healthy subjects (age: 59.2±8.3 yr)	Peritoneal dialysis for 26.56±23.27 months	a. ↑ RBC: 10.8±3.8 Plasma: ↑ 74.5±20.5 b. RBC: 4.8±2.6 Plasma: 46.6±16.1 (nmol/g Hb or prot)		a.RBC: ↑0.039±0.014 Plasma: 0.011±0.006 b.RBC 0.012±0.011 Plasma: 0.009±0.005 (μmol/g Hb or prot)	a.RBC: 6.40±1.36 Plasma: ↓ 1.53±0.69 b.RBC: 4.97±0.78 Plasma: 2.07±0.20 (μmol/g Hb or prot)		a.RBC: ↑48.7±11.5 Plasma: 5.02±1.36 b.RBC 36.1±8.5 Plasma: 5.50±0.79 (U/g Hb or prot)			
(Choi et al., 2011)	a. 18 patients (age 48.1±16.4 yr) b. 18 patients (age 52.0±17 yr) c. 16 healthy subjects (age 48.9±15.4 yr)	a. Peritoneal dialysis (PD) for 99.8±39.4 months, 1.7x/wk b. Typical HD for 94.3±43.5 months, 3x/wk							a. ↑12.2±3.5 b. 13.0±9.4 c. 4.5±0.9 (RLU)		
(Gonzalez-Diez et al., 2012)	a. 25 patients b. 15 patients (age: -)	a. Haemodialfiltration (HFR) for 1 year b. HD with polysulfone membrane for 1 year	a. ~19 b. ~17 (μM)	a. ~2.2 b. ~2.2 (nmol/mg prot)		a. ~1 b. ~0.9 (μmoles/g)	a. ~2100 b. ↓~1400 (U/g prot)	a. ~42 b. ~34 (U/g prot)		a. ~18 b. ~25 (U/g prot)	a. ~4 b. ~4 (mM Trolox)

TBARS: Thiobarbituric acid reactive substances ; Ox-LDL: oxidized Low Density Lipoprotein ;GSSG: oxidized glutathione; GSH: reduced glutathione ; SOD: superoxide dismutase ; GPx: glutathione peroxidase ; XO: Xanthine Oxidase ; CAT: Catalase ; TAC: Total Antioxidant Capacity; yr: years; ↑: versus healthy, ↓: versus healthy, ~: values were estimated according to figures.

### 3.2.7 Interventions in PD

Imani *et al* (Imani et al., 2009), evaluated the effects of soy consumption (28 g/day) for 8 weeks on oxidative stress in PD patients. Results indicated no changes in serum ox-LDL between soy and control group of PD patients, however an improvement in thrombosis risk was reported. The fact that the 78% of the specific peritoneal dialysis patients received Vitamin C and/or E, could explain the lack of a measurable effect. We do consider however that such dietary interventions as well as antioxidant interventions of adjunct pharmaceuticals need further examination (see below).

Overall, based on the limited data reviewed so far, PD also appears to be associated with increased oxidative stress, with the Choi *et al* study (Choi et al., 2011) showing PD patients to have as high XO activity as HD patients and the Canestrari *et al* study (Canestrari et al., 1995) showing not only increased oxidative stress but also evidence of reductions in antioxidant capacity, compared to controls. As XO is a superoxide producing enzyme its inhibition has been viewed as a way to reduce CVD risk (e.g. via allopurinol, (Dawson & Walters, 2006). To circumvent possible allopurinol's toxicity in renal insufficiency patients careful screening and tolerance protocols are needed (Jung et al., 2015). A new non-purine alternative, febuxostat, has been shown to reduce serum levels of uric acid and levels of a marker of DNA oxidation (8-oxo-dG) following 6 month administration in HD patients (Akimoto et al., 2014).

In non-CKD patients, soy consumption has been reported to improve redox status (for example in gestational diabetes (Jamilian & Asemi, 2015)). Notably, the combination of soy milk with *Lactobacillus plantarum* was recently shown not only to improve redox status, vs consumption of plain soy milk, but also to exert a promising epigenetic effect on the DNA repair capability of type II diabetic patients (Hariri et al., 2015). Dietary antioxidants may play a role even before disease diagnosis, whether independently or as a component of a generally 'healthy lifestyle', as an increased albuminuria (a prognostic marker for kidney disease), has been found to be associated with reduced levels of diet-derived carotenoids in an indigenous Australia population (Rowley, O'Dea, Su, Jenkins, & Best, 2003). Such and other findings highlight the complexity and overarching effect on redox status introduced by diet. We suspect that whether early in the disease, or towards advanced stages (especially

when CKD patients face severe nutritional restrictions, but were at the same time, not only noxious but potentially beneficial antioxidant intermediates as well may accumulate (Himmelfarb et al., 2003)), the role of targeted nutritional aids to reduce systemic oxidative stress could prove crucial for the reduction of CVD and malignancies risk.

In conclusion it could be indicated that oxidative stress is implicated in the CKD pathophysiology and as the kidney function is getting worse, the redox status imbalance becomes more profound. Even at an early disease state, lipid peroxidation markers are elevated. This premature development sets perhaps the pace for accelerated atherogenesis, which appears to explain the profoundly negative CVD risk of end-stage patients. Thus early interventions to combat lipid peroxidation in pre-dialysis patients need to be considered.

At the end-stage, patients present both with overabundance of ROS and mostly compromised antioxidant capacity. HD therapy *per se* seems to contribute to the oxidant and antioxidant imbalance. Iron supplementation has a pro-oxidant role but appears largely balanced out by appropriate EPO supplementation. The choice of dialysis membrane and dialyzing fluid glucose content may modulate the pro-oxidant effect of HD treatment. Moreover, antioxidant supplements may ameliorate the oxidative stress biomarkers by enhancing antioxidants defense, however mode of administration (i.e. intravenous for Vitamin C) should be carefully considered for efficacy. Vitamin E supplementation has been so far shown to be ineffective in HD patients, however small studies testing NAC and L-carnitine reported promising results.

Peritoneal dialysis did not appear to have a clear advantage over HD treatment, despite the biocompatibility advantage, while HFR may better preserve some antioxidant molecules. However the small number of studies in PD and HFR do not allow for safe conclusions and further work is needed to clarify the role of dialysis modality on redox status of ESRD patients.

### 3.3 The role of Oxidative stress in CKD-Skeletal Muscle levels

It is widely known that skeletal muscle activity generates ROS. Although high levels of ROS can damage cellular components, physiological levels of ROS play an important role in cells function including the regulation of cell signaling pathways, control of gene expression and modulation of muscle force production (Droge, 2002; Powers, Duarte, Kavazis, & Talbert, 2010; Reid, 2001a, 2001b). Several sources of endogenous and exogenous sources of ROS generation in skeletal muscle have been introduced.

Regarding endogenous sources, mitochondria are thought to produce ROS by a leak of single electron in the respiratory chain in the mitochondrial inner membrane of the contracting muscle cells. NADPH oxidases (NOX), which are transmembrane proteins in the transverse tubules and the sarcoplasmic reticulum transport electrons across biological membranes to reduce oxygen to superoxide or H<sub>2</sub>O<sub>2</sub> (Bedard & Krause, 2007; Brandes, Weissmann, & Schroder, 2014) and contribute to the cytosolic ROS production in skeletal muscle. Mitochondria and NADPH oxidase produce ROS in skeletal muscle both at rest and during contractile activity with NADPH oxidase to produce ROS to a larger extent than mitochondria (Shkryl et al., 2009; Xia, Webb, Gnall, Cutler, & Abramson, 2003). Moreover, xanthine oxidase is present in the cytosol of skeletal muscle and catalyzes the hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid (Harrison, 2002) During contraction xanthine oxidase contributes to lipid peroxidation, protein oxidation and muscle cell damage via the production of ROS. Finally, phospholipase A2 enzymes (PLA2) and myostatin is capable of signaling ROS production (Sriram et al., 2014; Zuo, Christofi, Wright, Bao, & Clanton, 2004).

Additionally, it has been shown that ROS are also produced from non-muscle sources disturbing muscle redox status and leading to tissue damage. Muscle injury, often observed during strenuous exercise, activates neutrophils and macrophages and although this process is necessary for fiber regeneration, it also results in ROS releasing (oxidative burst) (Moylan & Reid, 2007; Peake & Suzuki, 2004).

It is suggested that oxidative stress enhances the catabolic state and accelerates muscle atrophy (Moylan & Reid, 2007). The contractility of the remaining muscle and the sarcomeric protein expression are also affected by the imbalance of redox status.

Several studies have observed that oxidative stress causes acute and long-term effects on contractility. As an acute effect it is regarded the decrease in  $\text{Ca}^{2+}$  sensitivity which contributes to muscle fatigue, with reversible characteristics though. On the opposite, irreversible alterations in gene and protein expression or damages in proteins and lipids are considered as long-term effects (Lamb & Westerblad, 2011).

Regarding acute effect of ROS on muscle contractility, the reduced myofibrillar  $\text{Ca}^{2+}$  sensitivity and/or sarcoplasmic reticulum  $\text{Ca}^{2+}$  release has been proposed to play an important role in muscle fatigue (Allen, Lamb, & Westerblad, 2008). Furthermore, the increase of NO donors during fatigue has a negative impact on myofibrillar  $\text{Ca}^{2+}$  sensitivity in fast-twitch muscle fibers (Lamb & Westerblad, 2011). However, such a decrease in myofibrillar  $\text{Ca}^{2+}$  sensitivity observation was not noticed in slow-twitch fibers (Spencer & Posterino, 2009). Reardon & Allen (Reardon & Allen, 2009) have also observed that iron is capable of enhancing ROS production in skeletal muscle at high temperature.

It is widely accepted that actomyosin complex is essential for force generation in skeletal muscle. ROS can acutely affect its structure and functionality with negative results to the contractility. Prochniewicz *et al* (Prochniewicz *et al.*, 2008) found that exposure to low or high concentrations of hydrogen peroxide (5 or 50 mM) reduces maximum force and velocity of contractions. More specifically, the exposure to high concentrations of peroxide can cause oxidative damage of methionines which are contained in the heavy and essentially light chain of myosin. As a result, due to the irreversible loss of calcium regulation of force, a reduction of maximum force and velocity of contraction was observed (Prochniewicz *et al.*, 2008). Moreover, changes in the expression of myosin heavy chain in uremic animals have been reported (Taes *et al.*, 2004).

Besides the effects on contractile kinetics, oxidative stress also contributes to modulation of several signaling pathways such as calcium, phospholipases, phosphatases, serine/threonine kinases and protein tyrosine kinases (Jackson *et al.*, 2002) These modulation leads to changes in gene expression, cell function, metabolism and damage. Oxidative stress is accompanied by an increase in protein loss and muscle atrophy. Specifically, protein complexes such as NF- $\kappa$ B and FOXO



are activated via excessive ROS and in turn activate two muscle-specific E3 ubiquitin ligases, Muscle RING Finger 1 (MuRF-1) and Muscle Atrophy F-box (MAFbx, also known as Atrogin-1) (Gumucio & Mendias, 2013). Particularly, MuRF-1 and MAFbx have been shown to over-express in muscle atrophy (Bodine et al., 2001; Brooks & Myburgh, 2014; Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001) and degrade several proteins such as myosin light chains 1 and 2, myosin heavy chain, titin, nebulin, troponin and myosin-binding protein C (Cohen et al., 2009; Witt, Granzier, Witt, & Labeit, 2005).

Nonetheless, in many chronic inflammatory diseases, such as CDK, muscle weakness runs parallel with, and in part independently of muscle atrophy. In populations studied so far, although absolute strength is reduced, force normalized for loss of muscle mass remains unchanged. For example, ageing human populations experience the reduction in muscle strength that exceeds the loss of muscle mass (Goodpaster et al., 2006; Hairi et al., 2010). Studies with skinned fibers of patients with chronic obstructive pulmonary disease (Levine et al., 2003; Ottenheijm et al., 2005) or chronic heart failure (Szentesi et al., 2005) revealed reduced muscle force production that is independent of muscle mass loss.

Circulating pro-inflammatory mediators such as interleukin-6, C-reactive protein, sphingomyelase and tumor necrosis factor- $\alpha$  (TNF) found elevated in patients with chronic diseases and associated with muscle weakness (Cicoira et al., 2001; Doehner et al., 2007; Toth, Ades, Tischler, Tracy, & LeWinter, 2006; Yende et al., 2006), with TNF to be the most strongly implicated. Studies have shown that chronic exposure to TNF promotes decreased specific force and muscle weakness (X. Li et al., 2000; Tisdale, 2008) which is not entirely attributed to loss of muscle mass. It has been suggested that TNF has a direct effect on muscle by altering myofibrillar function with troponin, myosin heavy chain, or tropomyosin to constitute potential molecular targets of TNF/TNFR1 signalling. Experimental evidence suggested that muscle derived-oxidants are essential mediators of TNF/TNFR1-induced dysfunction. However the exact mechanism by which TNF depresses contractile function via oxidant activity has not been elucidated. Existing data supports that a cascade of potential signaling events takes place. There have been suggested several mechanisms for TNF-stimulated oxidants without though the predominant to have been identified. Additionally, the mechanism by which oxidant activity depresses myofibrillar function remains also unknown. Perhaps the mechanisms involve direct reaction of

TNF-stimulated oxidants with myofilament proteins (Burke, Reisler, & Harrington, 1976; Putkey, Dotson, & Mouawad, 1993; Williams & Swenson, 1982) or activation of redox-sensitive kinases or phosphatases that modify the phosphorylation state of myofibrillar protein and thereby influence force (Andrade, Reid, & Westerblad, 2001). Other post-translational mechanisms may include carbonylation (Coirault et al., 2007), ubiquitination (Dalla Libera et al., 2005) and myosin degradation (Tikunov, Mancini, & Levine, 1996).

In the literature, several studies evaluated the role of oxidative stress in skeletal muscle using in vivo or in vitro methods (Powers et al., 2011). However, only three studies (Table 5) have examined the oxidative damage in human skeletal muscle of uremic patients and the outcomes derive only from those being in the end stage of the disease.

Lim *et al* (Lim, Cheng, & Wei, 2002), found statistically significant elevation in the levels of protein carbonyls and malondialdehyde (MDA) in skeletal muscle of uremic patients, compared to healthy subjects, reflecting increased protein and lipid oxidation. It is suggested by the authors that oxidative damage in uremic patients is generated due to the increase of inflammatory cytokines, which is supported by the hemodialysis and the uremia per se. Oxidative damage of proteins may result in alterations in amino acid sequence, accumulation of modified proteins, carbonylation and rapid degradation. Taking into consideration that skeletal muscle contains high levels of fibrillar proteins, their oxidative modifications play a key role in the impaired structure and functionality and the emergence of uremic myopathy. Oxidative damage of membrane phospholipids results in the loss of the selective permeability and functional integrity of membranes, exposing them to damage.

According to another study of Lim *et al* (Lim, Ma, et al., 2002), it was observed that the average content of lipid peroxides and protein carbonyls in mitochondrial membranes of skeletal muscle of uremic patients were significantly higher than that of aged-matched healthy subjects. Additionally, oxidative damage to total cellular DNA was observed, suggesting not only a deterioration of mitochondrial function but also, an overall damage to the regenerative and bioenergetics capacity of skeletal muscle of uremic patients. Last but not least, increased mitochondrial DNA deletions was found in the skeletal muscle of ESRD patients compared with healthy subjects, attributed by the authors to the uremic toxins and the impaired antioxidant system of ESRD patients. These alterations in the genetic code of skeletal muscle may

contribute to the dysfunction of mitochondrial energy metabolism with negative effects for uremic patients (Lim, Cheng, & Wei, 2000).

On the other hand, Crowe *et al* (Crowe et al., 2007), found decreased levels of MDA and increased concentration of total glutathione in the skeletal muscle of patients with ESRD on HD compared to healthy subjects. Also, no differences in protein thiols content, oxidized glutathione (GSSG) concentrations, superoxide dismutase (SOD) and catalase activities between two groups were observed, concluding that muscle atrophy observed in these patients is not related with oxidative stress.

As it is noticed, there are conflicting results between the studies (Table 3.5) dealing with the evaluation of redox status indices in skeletal muscle of patients with CKD. If one takes into consideration the age difference in the patients of the studies, can conclude that younger patients have a better redox and mitochondrial status since they had spent less time on dialysis therapy. This could be an explanation why patients in Crowe *et al* study did not exhibit deteriorated muscle redox status indices. Furthermore, adaptation of several antioxidant mechanisms could be an additional factor for the conflicting results between the studies.

**Table 3.5.** Markers of oxidative stress in skeletal muscle tissue of uremic patients

Reference	Markers of oxidative stress in muscle tissue of uremic patients						
	Total glutathione nmol/mg protein	GSSG nmol/mg protein	SOD U/mg protein	MDA nmol/mg protein	CAT U/mg protein	PC nmol /mg of protein	Thiols nmol/mg protein
(Lim, Cheng, et al., 2002)	-	-	-	0.065±0.009 ↑	-	3.78±0.14 ↑	-
(Lim, Ma, et al., 2002)	-	-	-	23.76±6.06 ↑	-	24.9±4.00 ↑	-
(Crowe et al., 2007)	≈24 ↑	≈2.6	≈20	≈0.28 ↓	≈11 ↓	-	≈79
Reference	Markers of oxidative stress in muscle tissue of healthy controls						
(Lim, Cheng, et al., 2002)	-	-	-	0.043±0.005	-	2.97±0.28	-
(Lim, Ma, et al., 2002)	-	-	-	7.67±0.95	-	3.78±0.14	-
(Crowe et al., 2007)	≈5	≈3.3	≈27	≈0.52	≈34	-	≈60

GSH, glutathione; GSSG, oxidized glutathione ;SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; PC, protein carbonyl; Thiols, protein thiol content Arrows indicate statistically significant differences reported by authors

### 3.4 The practicality of animal models

The progressive and irreversible loss of renal function over a period of months or years is of diverse etiologies including metabolic syndrome, family history, environmental factors, and in many cases unidentified causes (Kurella, Lo, & Chertow, 2005). The disease is characterized by a variety of complex alterations in many organs and tissues making CKD a difficult task for medical and scientific approach.

Several animal models of CKD have been introduced in order to overcome the confounding factors of human disease by studying solely mechanisms in a limited timespan (Becker & Hewitson, 2013). Using such experimental models, researchers have the opportunity to understand the physiology and pathophysiology of the disease, to examine potential novel therapies, or to improve the existing ones (Becker & Hewitson, 2013). Depending on the mechanism, or the system of interest, CKD can be induced by *in vitro* or *in vivo* models and the choice of the model is of crucial role for the outcomes of each study. *In vitro* models are of small utility while results may vary depending on the cell type examined and therefore studies examined this kind of models are limited (H. C. Yang, Zuo, & Fogo, 2010). On the other hand, *in vivo* models are of larger interest while outcomes have a better reflection in *in vivo* physiology. As a result, a lot of animal models of CKD have been introduced such as vascular injury models, genetically engineered models, spontaneous models or immune and non-immune models (H. C. Yang et al., 2010). Non-immune models can be induced in many ways including surgical intervention called nephrectomy and it represents an animal model of CKD of known etiology. Surgical manipulations are widely performed to mimic chronic kidney human disease by reducing renal mass and to test therapeutic approaches.

Another critical factor for animal models is the choice of the right animal. There has been introduced a variety of species (mice, rats, rabbits, dogs) as animal models of CKD, but the right selection depends on what particular aspect of the disease is going to be reproduced each time. Murine models of kidney disease are increasingly preferred because of their small size, their low cost housing and the genetically

defined strains (Anders & Schlondorff, 2000). Furthermore, rodent models, remain the closest to human disease species (Becker & Hewitson, 2013).

New Zealand rabbits have been widely used for surgically induced CKD. One of these approaches was developed in 1982 by a two – step partial nephrectomy (Gotloib et al., 1982). First partial nephrectomy of left kidney was induced by electrocauterization keeping 1/3 of the kidney functional and after an interval of two weeks, a total removal of the right kidney was performed through a flank incision and by extraperitoneal approach. In this way the authors established a reliable animal model for investigating the metabolic complications of continuous ambulatory peritoneal dialysis (CAPD). This surgical approach that aims reproducing CKD by reduction of renal mass has been achieved by using different protocols and in various animal models (Dobbie, 1993; Gotloib et al., 1982; L. J. Ma & Fogo, 2003; L. J. Ma et al., 2005; Oreopoulos, Balaskas, Rodela, Anderson, & Oreopoulos, 1993).

Our research interests focus on uremic myopathy. Therefore, we chose the animal model of White New Zealand rabbit (WNZ) that can provide us the necessary experimental material – blood and muscle- for a variety of experiments, including biochemical, molecular analysis and mechanics respecting the “Three R’s” (Guide for the Care and Use of Laboratory Animals).

### **3.5 Conclusions and Unanswered Questions**

From the current literature review it can be concluded that the redox status of uremic patients have been widely studied. Although a variety of possible factors has been implicated, the results are usually controversial and there is not yet a clear explanation about the exact mechanism underlying redox status disturbance in CKD in blood as well as in skeletal muscle of patients.

As far as redox status evaluation in blood is concerned, studies revealed that oxidative stress is implicated in CKD pathophysiology and as the kidney function is getting worse, the redox status imbalance becomes more profound. Even at an early disease state lipid peroxidation markers are elevated. This premature development sets perhaps the pace for accelerated atherogenesis which appears to explain the profoundly negative CVD risk of end-stage patients. At the end-stage, patients present both with overabundance of ROS and mostly compromised antioxidant capacity and HD *pes se* seems to contribute to the oxidant and antioxidant imbalance. Iron

supplementation has a pro-oxidant role but appears largely balanced out by appropriate EPO supplementation. The choice of dialysis membrane and dialyzing fluid glucose content may modulate the pro-oxidant effect of HD treatment. Moreover, antioxidant supplements may ameliorate oxidative stress levels by enhancing antioxidants defense, however mode of administration (i.e. intravenous for Vitamin C) should be carefully considered for efficacy. Vitamin E supplementation has been so far shown to be ineffective in HD patients, however small studies testing NAC and L-carnitine reported promising results.

Peritoneal dialysis did not appear to have a clear advantage over HD treatment, despite the biocompatibility advantage, while HFR may better preserve some antioxidant molecules. However the small number of studies in PD and HFR do not allow for safe conclusions and further work is needed to clarify the role of dialysis modality on redox status of ESRD patients.

CKD patients are affected by multiple concomitant conditions like diabetes, dyslipidemia, and hypertension which are all associated with oxidative stress. The presence of CKD appears to further enhance the oxidative stress independently from the underlying conditions. Increasing evidence suggests that oxidative stress is a plausible independent cardiovascular risk factor in CKD. A more comprehensive study combining the simultaneous evaluation of a variety of oxidative stress and biochemical status indices should be done in order to identify these biomarkers that could be used in clinical practice to monitor the disturbed redox and lipid profile in CKD patients. More early- stage research is necessary in order to identify all these mechanisms from the early beginning where several intervention and therapies may delay, or possibly halt, the progression of the disease

Regarding skeletal muscle, the few studies that examined the effects of CKD on skeletal muscle redox status, do point to a possibly important role for oxidative stress in uremic myopathy. It is not known if hypothesized oxidative stress mediated effects on muscle function are more of an acute or a chronic nature. In vitro studies however show clearly that oxidative stress does have a role either via chronic, protein and other modifications or acute contractility effects. If anything these few studies and the peripheral literature highlight the need for a systematic study of the disease mechanisms affecting skeletal muscle performance in CKD.

As long as great unknowns remain on the mechanisms and modulation of uremic myopathy, which leads to debilitation and premature death, progress in this new epidemic is the least slowed down. It is suggested that more muscle research should be done on pre-dialysis stages including work on the role of oxidative stress. This would allow researchers to decipher early changes, and perhaps identify susceptible individuals for accelerated muscle loss, before moving into the end-stage situation which on its own has detrimental effects on muscle status.

Last but not least, judging from the above results, it is further confirmed the difficulties faced by the researchers in the field of CKD. A variety of confounding factors such as years in dialysis, comorbidities, pharmaceuticals, gender, nutritional status, physical activity levels can affect muscle and blood redox and biochemical status and accelerate or retard the disease manifestation. To answer such questions it was important to use methodology that avoids all those confounding factors which are unavoidable when studying patients. This issue can be satisfactorily addressed by employing an animal model mimicking chronic renal insufficiency.

Thus the current PhD thesis will try to cover the lack of knowledge answering to all these unanswered questions related to oxidative stress and CKD. Is there any oxidative stress damage or reduction in the antioxidant capacity in the early stages of the disease and which of the two becomes critical first? Do all these possible redox status disturbances influence biochemical profile in the early stages of CDK? What about the redox status of skeletal muscle? Is there any difference in redox status between two different types of skeletal muscle? Is this possible disturbance muscle type specific? What about biochemical status of skeletal muscle in the early stages of the disease? Is there any possible relationship between the changes in blood and muscle redox status between the two groups as a result of uremia?



## **4. Research Papers**

## **Research paper 1: Effects of CKD on blood redox status**

## Abstract

**Introduction:** It has been suggested that oxidative stress constitutes the basis of several pathologies encountered in CKD. Although the progression of CKD is associated with the harmful effects of oxidative stress, its role has not yet been fully clarified during the different stages of the disease

**Aims:** The aim of this study was to evaluate the systemic effects of CKD in redox status indices using an animal model of CKD and to identify which one of its components, defense capacity or ROS overproduction, becomes critical first.

**Methods:** We used an animal model (partial nephrectomy) of renal disease in New Zealand white female rabbits. Surgery and euthanasia (after 3 months) protocols were approved by the ethic committee of the University of Thessaly. Renal insufficiency was induced by removal of the right kidney and partial nephrectomy of the left kidney (Uremic group, N=9). Control animals underwent sham operation (Control group, N=6). Blood samples were collected and analysed for Uric acid, Glutathione Reduced (GSH), Glutathione Oxidized (GSSG), GSH/GSSG ratio, Total Antioxidant Capacity (TAC), Catalase Activity (CAT), Protein Carbonyls (PC) and Thiobarbituric Acid Reactive (TBARS). Urea, creatinine, total protein and hemoglobin levels were also evaluated.

**Results:** GSH concentration was significantly higher in the Uremic group ( $17.50 \pm 1.73 \mu\text{mol/g protein}$ ) compared to Control ( $12.43 \pm 1.01 \mu\text{mol/g protein}$ ),  $p=0.033$ . TBARS concentration tended to be higher in the Uremic group ( $7.03 \pm 0.81 \text{ nmol/ml}$ ), compared to Control ( $5.12 \pm 0.42 \text{ nmol/ml}$ ),  $p=0.060$ . No significant differences were found in the rest of redox status indices evaluated in blood of Control and Uremic groups ( $p>0.05$ ).

**Conclusion:** Evidence of early oxidative stress appears in the blood of our model of a pre-dialysis stage of CKD. As an adaptive response the levels of GSH, an antioxidant molecule, increased in order to keep redox homeostasis stable. However the tendency for increased lipid peroxidation (TBARS) is probably indicative of premature development of cardiovascular problems and atherogenesis in pro dialysis patients.

Thus early interventions to combat lipid peroxidation in these early stages may need to be considered.

## Introduction

The term redox status described the dynamic balance between oxidants (or pro-oxidants) and antioxidants and is considered a major factor for cellular homeostasis. Under physiological conditions the equilibrium between generation and elimination of ROS is maintained by several complex mechanisms. Redox homeostasis can be disrupted due to a dysfunction of any of these mechanisms, resulting in an increased ROS production and a decreased scavenging capacity, a condition which is described as oxidative stress. ROS are highly reactive to proteins, membrane lipids, carbohydrates and nucleic acids, causing irreversible damages which affect cell survival and lead to degenerative disorders and aging. (Trachootham, Lu, Ogasawara, Nilsa, & Huang, 2008).

It has been suggested that oxidative stress constitutes the basis of several pathologies encountered in CKD, such as anemia, inflammation, fatigue, muscle waste, disuse atrophy, atherosclerosis and cardiovascular disease (Himmelfarb & Hakim, 2003).

Although the progression of CKD is associated with the harmful effects of oxidative stress, its role has not yet been fully clarified during the different stages of the disease. The large majority of the existing data are referred to outcomes coming from the end-stage renal patients (ESRP) undergoing HD treatment. HD treatment is suggested to be responsible for increased oxidative stress mostly due to the hemoincompatibility induced by the direct contact of blood with the dialysis membrane (Horl, 2002; Morena et al., 2000). As a result, the neutrophils are activated releasing ROS into the blood stream (Hodkova et al., 2005). Four main factors have been proposed to be responsible for the harmful effects of oxidative stress in patients under HD: the uremic milieu, the HD treatment *per se*, the hemoincompatibility of dialysis system and the concomitant drug treatment (Canaud et al., 1999). Additionally, the precise evaluation of oxidative stress in ESRP is complex, since a variety of confounding factors, such as years in HD, comorbidities and pharmaceuticals are implicated. To avoid the above confounding factors, an animal model of CKD may be employed to better mimic CKD patients being in the pre-dialysis stage of the disease.

The aim of this study was to evaluate the circulating redox status in animals with pre-dialysis stage of renal insufficiency and to identify which one of its components, defense capacity or ROS overproduction, becomes critical first.

## Methods

### Animals and Experimental design

New Zealand white female rabbits (young adult, N=15) with a body weight of approximately 3200 kg, were first acclimatized to the laboratory animal unit of Medical School (University of Thessaly, Greece) for 48 hours. The animals were housed in a controlled environment with stable conditions of room temperature (RT) (22–24°C) and lighting (12:12 h light-dark cycle). All rabbits were fed with a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum. All animal procedures, including surgery and euthanasia for this project were approved by the ethics committee of the University of Thessaly (decision 2-2/10-10-2012) and the scientific committee of the University Hospital of Larisa, Greece (decision 1/4-1-2012) and animals were under veterinary care, in accordance to the national directives for the care and the use of laboratory animals.

After acclimatization, surgical procedures were performed (sham operation for control animals - Control group and partial nephrectomy for experimental animals – Uremic group. Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87 % and 13 % respectively (proportion 6,69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg body weight of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anesthesia was achieved by a dose of propofol (10mg/kg BW). Three hours before the intervention, each animal had only access to water and not to food and its weight was measured on a precision scale. Animal temperature was maintained via a heating pad.

The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982 (Gotloib et al., 1982). For the Uremic group nine animals (N=9), underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy ( $\frac{3}{4}$ ) of the right kidney. For the Control group six age-matched animals (N=6) underwent sham operation. Twelve weeks after surgery, the animals were weighed and then sacrificed by injection of

sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, sample collections were done in a blind fashion.

## **Sample Preparation**

### ***Blood sampling and treatment***

Blood samples (5ml) were collected from the rabbits' heart and aorta by a heparinized syringe and were placed into ethylene diamine tetra-acetic acid (K<sub>2</sub>EDTA)-containing tubes (Vacutainer Plus Plastic K<sub>2</sub>EDTA; Becton Dickinson). For plasma collection, blood samples were centrifuged immediately at 1370 × g for 10 min at 4° C and the supernatant was carefully collected, aliquoted in multiple eppendorf tubes, stored at -80°C and thawed only once before analysis. The remained packed erythrocytes were lysed with 1:1 (v:v), distilled water, inverted vigorously, and centrifuged at 4000 × g for 15 min at 4° C. The supernatant, red blood cells (RBCs) lysate designated, collected, aliquoted, stored at -80° C and thawed only once before analysis. Finally, in order to obtain serum, another portion of blood sample (5 ml) was collected and placed into separate tubes containing clot activator, left for 20 min to clot at RT and centrifuged at 1,370 × g, at 4°C for 10 min. The supernatant was collected, aliquoted in eppendorf tubes, stored at -80°C and thawed only once before analysis.

## **Biochemical Analyses**

### ***Urea, Creatinine, Total protein and Hemoglobin determination***

Urea and creatinine concentrations in serum were determined with the colorimetric method using the commercially available kits (ab83362, Abcam) and (ab65340, Abcam) respectively, a 96-well microtiter plate and a programmable microplate reader (Biochrom, Asys Expert 96). Urea and creatinine concentrations in unknown samples were determined by comparison with the standard curves.

Total protein concentration in plasma and skeletal muscle (psoas, soleus) homogenates were determined spectrophotometrically using the bicinchoninic acid



(BCA) protein assay kit (Pierce). In plasma, total protein concentration was determined in order to estimate the final concentration of Protein Carbonyls.

Hemoglobin concentration in red blood cell lysate was determined using a commercially available kit (Dutch Diagnostics BV, Zutphen, The Netherlands), in order to estimate the final concentration of Glutathione (reduced and oxidized) and Catalase activity.

## **Evaluation of oxidative stress**

### ***Determination of Reduced Glutathione***

Reduced Glutathione (GSH) concentration was determined in RBCs lysate according to Rahman *et al.* (2006) (Rahman, Kode, & Biswas, 2006), using a 96-well microtiter plate and a programmable microplate reader (Biochrom, Asys Expert 96). Briefly, blood samples were deproteinized with 5% trichloroacetic acid (TCA) (1:1 v/v) centrifuged at  $16,000 \times g$  for 10 min and the supernatant was collected. The following reagents were added in order (all reagents in 0.1 M potassium phosphate buffer, pH 7.5, with 5 mM EDTA) to 96-well flat-bottom plate (CytoOone) in duplicate. 20  $\mu\text{L}$  of glutathione standard (0.103 to 26.4  $\mu\text{M}$ ) or blood sample, 120  $\mu\text{L}$  of freshly prepared DTNB (2 mg/3 ml), Glutathione Reductase (10 Units) mix solution (1v:1v) and 60  $\mu\text{L}$  of NADPH (2 mg/3 ml). The absorbance at 415 nm was measured every 30 s, for 3 min, at room temperature (RT). The rate of increase in absorbance per minute was calculated by linear regression. Glutathione concentration in unknown blood samples was determined by comparison with the standard curve.

### ***Determination of Oxidized Glutathione***

Oxidized Glutathione (GSSG) concentration was determined in RBCs lysate according to Giustarini *et al.* (2013) (Giustarini, Dalle-Donne, Milzani, Fanti, & Rossi, 2013), modified for using a 96-well microtiter plate and a programmable microplate reader. Blood samples were deproteinized with 5% trichloroacetic acid (TCA) (1:1 v/v) centrifuged at  $16,000 \times g$  for 10 min and the supernatant was collected. To avoid the rapid oxidation of GSH to GSSG, through the deproteination procedure, and the consequent overestimation of GSSG, the alkylating reagent N-ethylmaleimide (NEM) 310 mM was added upon collection of blood sample or the

tissue homogenization. This was extracted before the actual measurement with three volumes of dichloromethane DCM, carefully collecting the upper volume of the ensuing bilayer (typically 750  $\mu$ L of DCM for 250  $\mu$ L of deproteinized supernatant, in a 1.5-ml Eppendorf tube, vortexed 5 min at 800 rpm RT and centrifuged at 14,000  $\times$  g for 30 sec at 4<sup>o</sup> C). To measure GSSG the following reagents were added in order (all reagents in 0.1 M potassium phosphate buffer, pH 7.5, with 5 mM EDTA) to 96-well flat-bottom plate (CytoOone) in duplicate: 20  $\mu$ L of glutathione disulfide standard (0.103 to 26.4  $\mu$ M) or the sample to be assayed, 120  $\mu$ L of freshly prepared DTNB (2 mg/3 ml) and Glutathione Reductase (10 Units) mix solution (1v:1v) and 60  $\mu$ L of NADPH (2 mg/3 ml). The absorbance at 415 nm was measured every 30 s, for 3 min, at RT. The rate of increase in absorbance per minute was calculated by linear regression. Glutathione oxidized concentration in unknown samples was determined by comparison with the standard curve.

#### ***Determination of Glutathione Reductase activity***

Glutathione reductase (GR) activity was determined in RBCs samples according to Cribb *et al.* (1989) (Cribb, Leeder, & Spielberg, 1989), using a 96-well microtiter plate and a programmable microplate reader. To measure glutathione reductase, the following reagents were added in order (all reagents in 0.1 M sodium phosphate buffer, pH 7.5, with 1 mM EDTA) to 96-well flat-bottom plate (CytoOone) in duplicate: 150  $\mu$ L of 0.1 mM DTNB, 10  $\mu$ L of NADPH (10 mg/ml; 12 mM), and 20  $\mu$ L of reductase standard (0.015 to 0.50 U/ml) or the blood sample to be assayed. The reaction was initiated by the addition of 10  $\mu$ L of GSSG (1 mg/ml; 3.25 mM). For blank wells, no GSSG was added. The absorbance at 415 nm was measured every 30 s, for 3 min, at room temperature. The rate of increase in absorbance per minute was calculated by linear regression. Glutathione reductase in unknown blood samples was determined by comparison with the standard curve

#### ***Measurement of Total Antioxidant Capacity***

Total antioxidant capacity (TAC) was determined in plasma samples according to Janaszewska and Bartosz (2002) (Janaszewska & Bartosz, 2002), based on the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. DPPH stock solution (10mM) was prepared by dissolving 0.02g DPPH in 5ml of methanol and mix in the stirrer. The working solution was obtained by diluting stock solution 100 times with methanol. In 20  $\mu$ L of plasma, 480  $\mu$ L of 10 mM sodium potassium

phosphate (pH 7.4) and 500  $\mu\text{L}$  of 0.1 mM DPPH were added and incubated in the dark for 30 min at RT. The samples were centrifuged for 3 min at  $20,000 \times g$  and 900  $\mu\text{L}$  of the supernatant was transferred into a clean plastic cuvette. The absorbance was read at 530 nm using a spectrophotometer. TAC values were presented as mM of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H).

#### ***Determination of Catalase activity***

Catalase (CAT) activity was determined in RBCs lysate according to Aebi (1984) method (Aebi, 1984). 20  $\mu\text{L}$  of RBCs hemolysate was added to 2975  $\mu\text{L}$  of sodium potassium phosphate buffer 67 mM, pH 7.4 and the samples were incubated at  $37^\circ\text{C}$  for 10 min. 5  $\mu\text{L}$  of hydrogen peroxide 30% was added and the change in absorbance was immediately read at 240nm for 2 min. One unit of catalase is equal to 1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  decomposed/minute. Results were normalized to hemoglobin content in the sample (units/mg hemoglobin).

#### ***Spectrophotometric DNPH assay for protein carbonyls content determination***

Protein carbonyls in oxidized proteins were determined in plasma samples according to Fields and Dixon (1971) (Fields & Dixon, 1971). In 50  $\mu\text{L}$  of plasma, 50  $\mu\text{L}$  of 20 % TCA was added, incubated in the ice bath for 15 min and centrifuged at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$  and the supernatant was discarded. Afterwards, 500  $\mu\text{L}$  of 14 mM 2,4-dinitrophenylhydrazine (DNPH), in 2.5 N HCl, for the sample or 500  $\mu\text{L}$  of 2.5 N HCl for the blank was added to the pellet. Both samples were incubated in the dark at RT for 1 h, with intermittent vortexing every 15 min. Samples were centrifuged at  $15,000 \times g$  for 5 min. at  $4^\circ\text{C}$ . The supernatant was discarded and 1 mL of 10% TCA was added, vortexed, and centrifuged at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant was discarded, and 1 mL of ethanol-ethyl acetate (1:1 v/v) was added, vortexed, and centrifuged at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The washing step was repeated two more times. The supernatant was discarded, and 1 mL of 5 M urea (pH 2.3) was added, vortexed, and incubated at  $37^\circ\text{C}$  for 15 min. The samples were centrifuged at  $15,000 \times g$  for 3 min at  $4^\circ\text{C}$ , and the absorbance was read at 375 nm. Protein carbonyls values were obtained by using the molar extinction coefficient of 2,4- dinitrophenylhydrazine ( $22 \text{ mM}\cdot\text{cm}^{-1}$ ).

### ***TBARS determination***

Thiobarbituric-acid reactive substances (TBARS) were determined in plasma samples according to Buege and Aust (1978) (Buege & Aust, 1978). (Buege & Aust, 1978). In 100  $\mu\text{L}$  of plasma, 500  $\mu\text{L}$  of 35 % TCA and 500  $\mu\text{L}$  of 200 mM Tris- HCl (pH 7.4) were added and incubated at RT for 10 min. Afterwards, 1 ml of 2 M  $\text{Na}_2\text{SO}_4$  and 55 mM thiobarbituric acid (TBA) solution was added and incubated at 95° C for 45 min. The samples were cooled on ice for 5 min and were vortexed. 1 ml of 70 % TCA was added, vortexed and centrifuged at 15,000  $\times$  g for 3 min at 25° C. The absorbance of the supernatant was read at 530nm. A baseline absorbance was taken into account by running a blank along with all samples during the measurement. The calculation of TBARS concentration was obtained using the molar extinction coefficient of MDA (15600 mol/l).

### ***Uric acid determination***

Uric acid concentration was measured on a Clinical Chemistry Analyzer Z 1145 (Zafiroopoulos Diagnostica, Athens, Greece) using commercially available kits (Zafiroopoulos Diagnostica). 6  $\mu\text{L}$  of serum were added to 600  $\mu\text{L}$  of working reagent, samples were incubated for 1 min at 37° C and the absorbance was read at 340 nm.

All materials for oxidative stress assays were purchased from Sigma (St. Louis, MO, USA)

### **Statistical Analysis**

Duplicate values were averaged. Data were analyzed using the commercially available statistical software package SPSS 22. The Shapiro-Wilk test was performed to initially test whether the data were normally distributed, as it was the case. Results are expressed as mean  $\pm$  SEM and 95% confidence intervals.

An independent t-test was conducted to examine whether there were any differences in blood redox status indices between Control and Uremic group. The significance level was set at  $p < 0.05$ .

## Results

### *Body weight and biochemical indices*

Both surgery procedures ( $\frac{3}{4}$  partial nephrectomy (Uremic group) and sham operation (Control group) were well tolerated by animals who presented with a normal after-surgery recovery. At the end of the twelve-week period post surgery, body weight ranged between 3,045-4,965 gr (with mean  $\pm$  SEM of  $3,728 \pm 336.47$  gr) for Control group and 1,970-4,585 gr (with mean  $\pm$  SEM of  $2,935 \pm 288.70$  gr) for Uremic group ( $p > 0.05$ ). Renal insufficiency in experimental animals, compared to Control, was reflected in raised blood of urea and creatinine levels. All results are represented in Table 4.1.1.

**Table 4.1.1.** Biochemical indices (Mean  $\pm$  SEM) in the Control and Uremic group. The exact statistical significance value P and the 95% Confidence Intervals are reported.

	CONTROL GROUP (n=6)	95% Confidence Interval		UREMIC GROUP (n=9)	95% Confidence Interval		P
		Lower Bound	Upper Bound		Lower Bound	Upper Bound	
Urea (mg/dl)	$38 \pm 4.3$	33.43	43.24	$60 \pm 11.52$	37.42	82.58	0.114
Creatinine (mg/dl)	$1.28 \pm 0.15$	1.11	1.45	$2.45 \pm 0.37$	1.72	3.19	0.018*
Total Protein (mg/ml)	$68.59 \pm 2.12$	64.44	72.75	$67.89 \pm 2.28$	63.41	72.37	0.825
Hemoglobin (mg/dl)	$11.08 \pm 0.92$	10.33	11.81	$9.80 \pm 0.30$	78.22	11.77	0.368

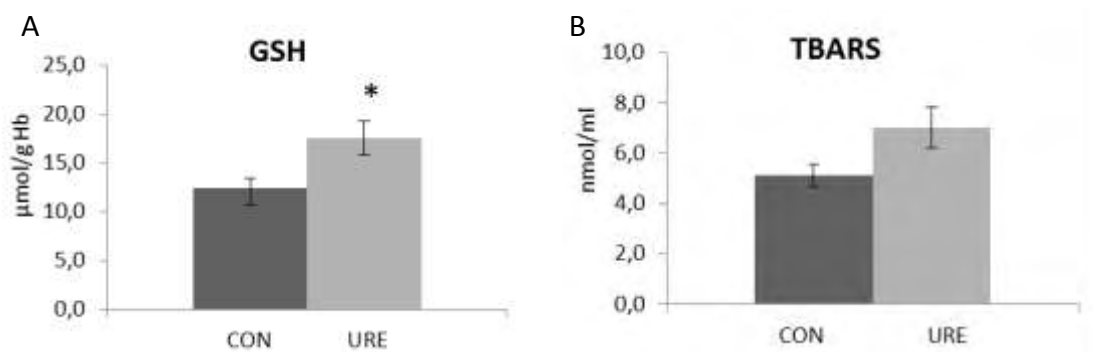
### *Blood oxidative stress analysis*

GSH concentration was significantly higher in the Uremic group compared to Control  $t(9) = -2.071$ ,  $p = 0.033$ . TBARS concentration tended to be higher in the Uremic group compared to Control ( $p = 0.080$ ) (Figure 1). No significant differences were found in the rest of redox status indices evaluated in blood of Control and Uremic groups ( $p < 0.05$ ), as presented in Table 4.1.2. GSH and TBARS levels are presented also in Figure 4.1.1.

**Table 4.1.2.** Blood redox status indices (Mean  $\pm$  SEM) in the Control and Uremic group. The exact statistical significance value P and the 95% Confidence Intervals are reported.

BLOOD	CONTROL GROUP (n=6)	95% Confidence Interval		UREMIC GROUP (n=9)	95% Confidence Interval		P
		Lower Bound	Upper Bound		Lower Bound	Upper Bound	
Uric Acid (mg/dl)	1.39 $\pm$ 0.25	0.907	1.886	1.93 $\pm$ 0.38	1.18	2.67	0.263
GSH ( $\mu$ mol/g protein)	12.43 $\pm$ 1.01	10.45	14.41	17.50 $\pm$ 1.73	14.12	20.88	0.033*
GSSG ( $\mu$ mol/g protein)	0.027 $\pm$ 0.006	0.016	0.039	0.048 $\pm$ 0.010	0.028	0.067	0.110
Ratio (GSH/GSSG)	481 $\pm$ 53	376	586	425.28 $\pm$ 61.19	305.36	545.21	0.511
GR Reductase (U/g protein)	176.40 $\pm$ 25	127.39	225.42	153.1 $\pm$ 15.84	122.04	184.15	0.425
TAC ( $\mu$ mol DPPH/ml)	0.786 $\pm$ 0.033	0.719	0.852	0.759 $\pm$ 0.041	0.679	0.838	0.611
CAT (U/mg protein)	342.02 $\pm$ 17.69	307.35	376.68	303.63 $\pm$ 15.63	273.01	334.26	0.131
PC (nmol/mg protein)	0.603 $\pm$ 0.09	0.427	0.780	0.620 $\pm$ 0.066	0.491	0.748	0.888
TBARS (mmol/ml)	5.12 $\pm$ 0.42	4.29	5.94	7.03 $\pm$ 0.81	5.44	8.62	0.060

GSH: Reduced Glutathione GSSG: Oxidized Glutathione TAC: Total Antioxidant Capacity, CAT: Catalase, PC: Protein Carbonyls, TBARS: Thiobarbituric Acid Reactive Substances, \* statistical significance between Control and Uremic group,  $p < 0.05$



**Figure 4.1.1.** Indicative blood redox status indices (Mean  $\pm$  SEM) in the Control and Uremic group. A: GSH and B: TBARS concentration in blood of Control and Uremic group, \*  $P < 0.05$  compared to Control

No significant differences were found in the rest of redox status indices evaluated in blood of Control and Uremic group ( $p < 0.05$ ), as presented in Table 4.1.2.

## Discussion

This study was performed to analyze a range of redox status indices in blood using a rabbit model of CKD. Our major findings were that GSH concentration was significantly higher in the erythrocytes of the Uremic group compared to the Control and that there was a tendency for an increase in TBARS concentration in the plasma of Uremic group compared to Control.

GSH is a primary antioxidant molecule which belongs to the endogenous defense against ROS and its role is critical for the cellular redox environment (Small, Coombes, Bennett, Johnson, & Gobe, 2012), since it is the most abundant non-protein thiol that defends against oxidative stress (Lu, 2013). Specifically, GSH is synthesized in the cytosol in virtually all cells (highest concentrations are in the liver) from its constituent amino acids, glutamate, cysteine and glycine via the sequential action of two ATP-dependent cytosolic enzymes, namely gamma-glutamylcysteine synthetase ( $\gamma$ -GCS) and glutathione synthetase (GSH-S) (Anderson & Meister, 1983). With regard to red blood cells (RBCs), they can also synthesize GSH through the above procedure since they contain all the enzymes for its biosynthesis and a significant percentage of their GSH is produced de novo daily (Dass, Bermes, & Holmes, 1992). It exists in the thiol-reduced and disulfide-oxidized (GSSG) forms (Kaplowitz, Aw, & Ookhtens, 1985) but GSH is the predominant form and accounts for >98% of total GSH (Forman, Zhang, & Rinna, 2009). There are three GSH reservoirs in the eukaryotic cells. Most (80-85%) of the cellular GSH is in the cytosol, 10-15% is in the mitochondria and a small percentage is in the endoplasmic reticulum (C. Hwang, Sinskey, & Lodish, 1992; Meredith & Reed, 1982).

Measured GSH blood levels are thus, the result of the dynamic balance between GSH synthesis, its recycling from GSSG (by Gr-Reductase) and its utilization (by peroxidases, transferases, transhydrogenases and transpeptidases).

According to the literature (human studies), there are conflicting results regarding GSH concentration in CKD patients, which appear sometimes to depend on the severity of the disease (as reflected by urea and creatinine levels) and sometimes not. Thus, in moderately uremic pre-dialysis patients, in agreement to our observations, Bober et al showed higher levels of GSH in whole blood of CKD patients compared to age-matched healthy (Bober et al., 2010) (which increased

further in HD patients). However, in other studies a reduction in GSH levels in the whole blood of CKD patients has been observed compared to controls (Annuk, Zilmer, Lind, Linde, & Fellstrom, 2001; Ceballos-Picot et al., 1996; Sahni et al., 2012) reflecting a depletion in the antioxidant reserve. Alhamdani et al (Alhamdani, 2005) evaluated the glutathione biosynthetic pathway in advanced uremia and hemodialysis measuring GSH levels,  $\gamma$ -GCS and GSH-S activities in non-dialysis, haemodialysis and continuous ambulatory peritoneal dialysis patients. Significant decreases in GSH levels and  $\gamma$ -GCS activity but not GSH-S were observed in all groups of patients compared to healthy individuals. Thus, low activity of  $\gamma$ -GCS, the rate limiting enzyme of GSH biosynthesis may have negatively affected *de novo* synthesis of GSH in those patients.

GSH outcomes remain also contradictory in patients undergoing HD therapy, where either lower (Drai et al., 2001; Lim, Cheng, et al., 2002; Schettler et al., 1998) or higher (Biasioli, Schiavon, De Fanti, Cavalcanti, & Giavarina, 1996; Bober et al., 2010; Stepniewska et al., 2006) GSH concentrations have been observed in the blood of HD patients compared to healthy individuals.

The increased levels of GSH concentration in some CKD patients could be attributed to the upregulation of its synthesis in response to a greater demand. The antioxidant function of GSH is accomplished by GSH peroxidase (GPx)-catalyzed reactions, which reduce hydrogen peroxide and lipid peroxide to water and alcohols respectively, as GSH is oxidized to GSSG. GSSG in turn is reduced back to GSH by Gr-Reductase at the expense of NADPH, forming a redox cycle (Lu, 2009). Overall, the increased GSH levels in our study could be the result of the cell's protective adaptive response to the uremic environment.

The latter appears more likely in our case given the almost doubling of blood GSSG in UREM (UREM levels being 1.77 fold of those of CON) which, without reaching statistical significance, indicated increased levels of hydrogen peroxide or lipid peroxides, similarly to human studies (Annuk et al., 2001). Nonetheless, catalase also reduces hydrogen peroxide to water but there was no statistical difference in the activity of the specific antioxidant enzyme between the Control and the Uremic group (rather activity tended to be lower in erythrocytes of the Uremic group). As it is known catalase is located in peroxisomes and, in contrast, GSH and GPx is found mainly in the cytosol (Baud et al., 2004). This subcellular compartmentalization is undoubtedly important for hydrogen peroxide detoxification. Based on the above, it



appeared that hydrogen peroxide scavenging was undertaken in a greater degree by the glutathione redox cycling mechanism than catalase in our CKD model. The significant increase of GSH in uremic blood samples can also explain the undisturbed TAC and carbonylation levels of blood proteins.

In our study animals followed the same diet, carefully designed not to tax the remaining kidney function, similarly to diet guidelines followed by patients, while providing balanced nutrients and minerals. However, dietary intakes can greatly influence GSH levels, and could explain the literature's conflicting reports with regards to human patients' GSH levels. As cysteine is the limiting amino acid for GSH synthesis, GSH concentration has been shown to be decreased by fasting, low-protein diets, or diets limiting in sulfur amino acids (Paterson & Juurlink, 1999). Several studies revealed that  $\alpha$ -Lipoic acid, a naturally occurring thiol compound, administration increases GSH levels in several cell types and tissues (Biewenga, Haenen, & Bast, 1997; Han et al., 1997; Paterson & Juurlink, 1999) and also restores intracellular GSH in several pathological conditions (Paterson & Juurlink, 1999). Moreover, selenium (Se) as an integral part of the enzyme GPx, plays a key role in GSH levels (Sunde & Hoekstra, 1980). In patient studies, while general clinical guidelines are the same, large differences in selenium or  $\alpha$ -Lipoic acid intake cannot be excluded which could account for the literature's conflicting results in GSH levels.

An issue sometimes overlooked is the sensitivity of GSH and GSSG levels to sample handling errors and possible methodological oversights. Major artifacts can occur during GSH and GSSG titration, with the most important one being a 5-15% oxidation of GSH during sample deproteination with acids. This artifact can lead to remarkable overestimation of GSSG (Rossi et al., 2002) and underestimation of total GSH. To support the purposes of this research we applied the thiol alkylating agent NEM at the preparatory stages to prevent the oxidation of GSH. In perusing the conflicting patient studies (Annuk et al., 2001; Bober et al., 2010; Ceballos-Picot et al., 1996; Sahni et al., 2012) it is not clear if such measures were taken except for the study of Ceballos- Picot et al., 1996 where GSSG concentration was determined after sample derivatization by adding 2-vinylpyridine to prevent GSH oxidation (Ceballos-Picot et al., 1996). Notwithstanding the above, it should be also noted that blood levels of GSH, as a 'systemic' marker, cannot reveal its origins. However, its increased levels in circulation confer an antioxidant advantage preventing or ameliorating vascular and other damage.

The increased (but not statistically significant) plasma TBARS levels by approximately 1.4 fold, in Uremic compared to Control samples, point to increased levels of lipid peroxidation, a process where oxidants, such as free radicals, assault lipids and especially their membranes (Ayala et al., 2014), leading to alterations of cell's quality and survival. For example, oxidatively modified low-density lipoprotein (ox-LDL) is regarded as a pathogenetic factor in atherosclerosis (Steinberg, 1997). Furthermore, lipid peroxidation negatively affects erythrocytes membrane integrity, playing a major role to their half-life shortening and thus, to the development of anemia (Peuchant et al., 1997). Papavasiliou et al (Papavasiliou et al., 2005) found statistically significant increased TBARS levels in plasma of pre-dialysis patients with stage 3-5 CKD, compared to healthy individuals. In the same study, patients on stages 1-2 CKD, exhibited a tendency for higher TBARS levels compared to healthy individuals. In contrast, stages 1-2 CKD patients, exhibited significantly lower MDA levels compared to the stages 3-5 CKD patients (Papavasiliou et al., 2005). Regarding HD patients, the large majority of studies reported increased TBARS levels in plasma compared to healthy individuals (Bober et al., 2010; Dimitrijevic et al., 2012; Guo et al., 2013; Haklar et al., 1995; Sakata et al., 2008; Sommerburg et al., 1998), reflecting extensive lipid peroxidation. Taking all the above into consideration together with our findings, it could be concluded that lipid peroxidation in CKD emerges even from the early stages and is getting worse during the progression of the disease.

## Conclusion

Evidence of early oxidative stress appears in the blood of our model of a pre-dialysis stage of CKD. As an adaptive response the levels of GSH, an antioxidant molecule, increased in order to keep redox homeostasis stable. On the other hand, there was a tendency for increased lipid peroxidation in plasma of Uremic rabbits as compared to sham-operated. This premature development sets perhaps the pace for accelerated atherogenesis, which appears to explain the markedly increased incidence of cardiovascular disease (CVD) in ESRD patients. Thus early interventions to combat lipid peroxidation in pre-dialysis patients may need to be considered.

## **Research paper 2: Effects of Chronic Kidney Disease on skeletal muscle redox status: the role of muscle type**

## Abstract

**Introduction:** Uremia affects skeletal muscle structure and function leading to muscle atrophy, muscle weakness, metabolic disorders, diminished exercise capacity and fatigue. Complex mechanisms that stimulate muscle dysfunction have been proposed, and oxidative stress may be implicated.

**Aims:** The aim of this study was to evaluate the effects of uremia on muscle redox status in a rabbit model of renal insufficiency and more specific in two different types of skeletal muscle, in the fast and glycolytic psoas and in the slow and oxidative soleus.

**Methods:** We used an animal model (partial nephrectomy) of renal disease in New Zealand white female rabbits. Surgery and euthanasia (after 3 months) protocols are approved by the ethic committee of the University of Thessaly. Renal insufficiency was induced by removal of the right kidney and partial nephrectomy of the left kidney (Uremic group, N=8). Control animals underwent sham operation (Control group, N=5). Psoas and soleus muscle samples were harvested, homogenized and analyzed for Protein Carbonyls (PC), Thiobarbituric Acid Reactive (TBARS), Glutathione Reduced (GSH), Glutathione Oxidized (GSSG), GSH/GSSG ratio, GR-Reductase activity, Total Antioxidant Capacity (TAC) and Catalase Activity (CAT). Total protein was also evaluated.

**Results:** No interactions were found between the two examined muscle types (psoas, soleus) and two groups (Control, Uremic) for all the redox status indices ( $p > 0.05$ ). Significant group main effects were found for PC in both muscle types with its concentration to be higher in the Uremic group (psoas:  $1.086 \pm 0.294$ , soleus:  $2.52 \pm 0.29$  nmol/mg protein) compared to the Control (psoas:  $0.596 \pm 0.372$ , soleus:  $0.929 \pm 0.41$  nmol/mg protein).

Significant muscle main effects were found for Total protein, PC, TBARS, GSH, TAC, CAT and GR-Reductase activity in both the Control and the Uremic group. Total protein and TAC concentrations were significantly lower in soleus compared to psoas muscle in both the Control and the Uremic group. Additionally, soleus demonstrated higher levels of TBARS and PC levels as well as higher GSH levels,

Catalase and GR-Reductase activities compared to psoas muscle in both the Control and Uremic group.

**Conclusion:** The increased PC levels in soleus in Uremic compared to Control samples, point to increased levels of sarcomeric protein carbonylation and this early countermeasure for protein oxidation could be of great importance for patients before they move into the end-stage where the negative effects on muscle status may become irreversible. Since carbonyl formation is associated with protein degradation and proteolysis, the increased levels seem to constitute an early stimulus to muscle atrophy observed in these patients during the progression of the disease.

Both oxidative damage and antioxidant response seems to be tissue-specific. Soleus muscle, which has greater number of mitochondria, by increasing ROS production it may be more exposed to ROS and their negative effects, but also that it develops an appropriate antioxidant capacity to withstand the oxidative load. In response to a further oxidative load due to renal insufficiency, uremic soleus appears to have further upregulated its defenses in comparison with uremic psoas.

Our results highlight the need for interventions early during disease progression in order to protect skeletal muscle quality and reach at the end-stage at the best possible functionality. How the mechanisms of oxidative stress in the pre-dialysis stage of CKD accelerates protein carbonylation and degradation remains to be determined.

## Introduction

Oxidative stress is characterized by an imbalance between pro-oxidants and antioxidants. Over the last few years it is increasingly accepted that patients with CKD experience increased oxidative stress. Moreover it has also been shown that these patients frequently suffer from easy fatigability and muscle weakness. All of these pathological parameters are collectively termed uremic myopathy (Campistol, 2002) which contributes to poor quality of life and high mortality.

Skeletal muscle *per se* is a source of ROS. It has been shown that muscle activity leads to a strong increase in ROS production (Powers & Jackson, 2008), especially in exercise (Alessio, Goldfarb, & Cutler, 1988; K. J. Davies, Quintanilha, Brooks, & Packer, 1982; Reid, Haack, et al., 1992; Reid, Shoji, Moody, & Entman, 1992). Oxidative stress mechanisms have been suggested to play a significant role in the conceivably related situations of disuse atrophy, age-related muscle atrophy and cancer cachexia (Jackman & Kandarian, 2004). However it remains unclear if oxidative stress plays a key role in the uremic myopathy commonly observed in CKD patients (Lim, Cheng, et al., 2002).

Several studies have indicated that CKD is characterized by modifications of cell energy metabolism leading to a reduction in muscle performance (Brautbar, 1983; Del Canale et al., 1986; Metcalf et al., 1983). It has been suggested that hemodialysis treatment and/or uremia *per se* contribute to an increase in molecular oxidative damage which in turn play a key role for loss of skeletal muscle functionality. Additionally, animal studies have shown that experimentally induced oxidative stress cause myofibrillar protein modification and degradation (Nagasawa et al., 1997) through alterations of its amino acid sequences. A variety of amino acid modifications exists with carbonyl formation to be considered as an early marker of protein oxidation. Arginine, proline and threonine are the most likely amino acids to form carbonyl derivatives (Reznick & Packer, 1994). Given that skeletal muscle contains high levels of fibrillar proteins and it becomes extremely susceptible to free radical oxidation with adverse outcomes.

Our hypothesis is that alterations of skeletal muscle function in CKD patients could be a consequence of the accumulation of oxidative damage to lipids and proteins that are exposed to the uremic milieu. The advance in methodologies for

evaluating oxidative stress will elucidate mechanisms and pathways which are responsible for redox status disturbances in skeletal muscle of CKD patients. However, most of the evidence of oxidative stress in skeletal muscle derives from studies in non-uremic populations (Haycock, Jones, Harris, & Mantle, 1996; Mecocci et al., 1999).

In CKD, as reviewed by us (Kaltsatou et al., 2015), so far only three studies have evaluated the role of oxidative stress in skeletal muscle of patients (Crowe et al., 2007; Lim, Cheng, et al., 2002; Lim, Ma, et al., 2002) undergoing hemodialysis. Two of three studies conducted by the same group indicated a possibly important role for oxidative stress in uremic myopathy via increased lipid peroxidation and protein carbonylation leading muscle cells into a catabolic state (Lim, Cheng, et al., 2002; Lim, Ma, et al., 2002). The authors also reported mitochondrial DNA mutations and overall oxidative damage to total cellular DNA. On the contrary, Crowe et al., 2007 found decreased MDA levels, increased t-GSH and no change in protein thiols, SOD and GSSG levels and CAT activity in skeletal muscle of uremic patients compared to healthy individuals, concluding that there is no relationship between muscle atrophy and uremia (Crowe et al., 2007).

The above discrepancies confirm that the precise evaluation of oxidative stress in ESRP is complex, since a variety of confounding factors, such as years in HD, comorbidities and pharmaceuticals are implicated. Thus there is a lack of data linking oxidative stress in skeletal muscle and its possible role in early stages of CKD.

The aim of the present study was to evaluate the effects of uremia on muscle redox status in a rabbit model of renal insufficiency and more specific in two different types of skeletal muscle, in the fast and glycolytic psoas and in the slow and oxidative soleus.



## Methods

### Animals and Experimental design

New Zealand white female rabbits (young adult, N=15) with body weight of approximately 3200 kg, were first acclimatized to the laboratory animal unit of Medical School (University of Thessaly, Greece) for 48 hours. The animals were housed in a controlled environment with stable conditions of room temperature (RT) (22–24°C) and lighting (12:12 h light-dark cycle). All rabbits were fed with a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum. All animal procedures, including surgery and euthanasia for this project were approved by the ethics committee of the University of Thessaly (decision 2-2/10-10-2012) and the scientific committee of the University Hospital of Larisa, Greece (decision 1/4-1-2012) and animals were under veterinary care, in accordance to the national directives for the care and the use of laboratory animals.

After acclimatization, surgical procedures were performed (sham operation for control animals - Control group and partial nephrectomy for experimental animals – Uremic group. Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87 % and 13 % respectively (proportion 6,69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg body weight of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anesthesia was achieved by a dose of propofol (10mg/kg BW). Three hours before the intervention, each animal had only access to water and not to food and its weight was measured on a precision scale. Animal temperature was maintained via a heating pad.

The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982 (Gotloib et al., 1982). For the Uremic group nine animals (N=9), underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy ( $\frac{3}{4}$ ) of the right kidney. For the Control group six age-matched animals (N=6) underwent sham operation. Twelve weeks after surgery, the animals were weighed and then sacrificed by injection of

sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, sample collections were done in a blind fashion.

### **Sample Preparation**

#### ***Skeletal muscle sampling***

Psoas and soleus muscle samples were harvested from Control (sham-operated) and Uremic group, were frozen immediately in liquid nitrogen, stored at -80° C and part-thawed once (for homogenization) before final analysis.

#### ***Skeletal muscle homogenization***

Tissues samples were thawed excised and kept chilled throughout homogenization. A weight portion of each muscle was washed several times with ice-cold normal saline and was placed into pre-chilled eppendorfs containing cold homogenization buffer (138 mM NaCl, 2,7 mM KCl, 1 mM EDTA, pH 7.4) and a mix of protease inhibitors (1µM aprotinin, 1µg/ml leupeptin and 1mM PMSF). Initial homogenization was achieved with an electrical homogenizer (MICCRA D-9) for 10 minutes with intermediate pauses of 10sec/20 sec. Then an ultrasound homogenizer (UP50H) was used for 1-2 minutes, with intermediate pauses as before. Homogenates were filtered through four layers of medical gauze to remove connective tissue debris, incubated for 10 m in at 4° C, and centrifuged at 10,000 X g for 10min again at 4° C. The supernatant was aliquoted in multiple portions and stored at -80° C.

### **Biochemical Analyses**

Total protein in muscle homogenates was determined spectrophotometrically using the bicinchoninic acid (BCA) protein assay kit (Pierce), in order to estimate the final concentration of Protein Carbonyls concentration.

### **Measurements of oxidative stress**

#### ***Determination of Reduced Glutathione***

Reduced Glutathione (GSH) concentration was determined in psoas and soleus homogenates samples according to Rahman *et al.* (2006) (Rahman et al., 2006), using a 96-well microtiter plate and a programmable microplate reader. Briefly, homogenate samples were deproteinized with 5% trichloroacetic acid (TCA) (1:1 v/v) centrifuged

at 16,000 X g for 10 min and the supernatant was collected. The following reagents were added in order (all reagents in 0.1 M potassium phosphate buffer, pH 7.5, with 5 mM EDTA) to 96-well flat-bottom plate (CytoOone) in duplicate. 20  $\mu$ L of glutathione standard (0.103 to 26.4  $\mu$ M) or homogenate sample, 120  $\mu$ L of freshly prepared DTNB (2 mg/3 ml), Glutathione Reductase (10 Units) mix solution (1v:1v) and 60  $\mu$ L of NADPH (2 mg/3 ml). The absorbance at 415 nm was measured every 30 s, for 3 min, at room temperature (RT). The rate of increase in absorbance per minute was calculated by linear regression. Glutathione concentration in unknown blood samples was determined by comparison with the standard curve.

#### ***Determination of Oxidized Glutathione***

Oxidized Glutathione (GSSG) concentration was determined in psoas and soleus homogenates samples according to Giustarini *et al.* (2013) (Giustarini et al., 2013), modified for using a 96-well microtiter plate and a programmable microplate reader. Homogenate samples were deproteinized with 5% trichloroacetic acid (TCA) (1:1 v/v) centrifuged at 16,000 X g for 10 min and the supernatant was collected. To avoid the rapid oxidation of GSH to GSSG, through the deproteination procedure, and the consequent overestimation of GSSG, the alkylating reagent N- ethylmaleimide (NEM) 310 mM was added during tissue homogenization. This was extracted before the actual measurement with three volumes of dichloromethane DCM, carefully collecting the upper volume of the ensuing bilayer (typically 750  $\mu$ L of DCM for 250  $\mu$ L of deproteinized supernatant, in a 1.5-ml microcentrifuge tube, vortexed 5 min at 800 rpm RT and centrifuged at 14,000 X g for 30 sec at 4<sup>o</sup> C). To measure GSSG the following reagents were added in order (all reagents in 0.1 M potassium phosphate buffer, pH 7.5, with 5 mM EDTA) to 96-well flat-bottom plate (CytoOone) in duplicate: 20  $\mu$ L of glutathione disulfide standard (0.103 to 26.4  $\mu$ M) or the homogenate sample, 120  $\mu$ L of freshly prepared DTNB (2 mg/3 ml): Glutathione Reductase (10 Units) mix solution (1v:1v) and 60  $\mu$ L of NADPH (2 mg/3 ml). The absorbance at 415 nm was measured every 30 s, for 3 min, at RT. The rate of increase in absorbance per minute was calculated by linear regression. Glutathione disulfide concentration in unknown homogenate samples was determined by comparison with the standard curve.

#### ***Determination of Glutathione Reductase activity***

Glutathione reductase (GR) activity was determined in psoas and soleus homogenates samples according to Cribb *et al.* (1989) (Cribb et al., 1989), using a 96-well microtiter plate and a programmable microplate reader. To measure glutathione reductase, the following reagents were added in order (all reagents in 0.1 M sodium phosphate buffer, pH 7.5, with 1 mM EDTA) to 96-well flat-bottom plate (CytoOone) in duplicate: 150  $\mu$ L of 0.1 mM DTNB, 10  $\mu$ L of NADPH (10 mg/ml; 12 mM), and 20  $\mu$ L of reductase standard (0.015 to 0.50 U/ml) or the homogenate sample to be assayed. The reaction was initiated by the addition of 10  $\mu$ L of GSSG (1 mg/ml; 3.25 mM). For blank wells, no GSSG was added. The absorbance at 415 nm was measured every 30 s, for 3 min, at room temperature. The rate of increase in absorbance per minute was calculated by linear regression. Glutathione reductase in unknown homogenate samples was determined by comparison with the standard curve

#### ***Measurement of Total Antioxidant Capacity***

Total antioxidant capacity (TAC) was determined in psoas and soleus homogenates samples according to Janaszewska and Bartosz (2002) (Janaszewska & Bartosz, 2002). In 20  $\mu$ L of homogenate, 480  $\mu$ L of 10 mM sodium potassium phosphate (pH 7.4) and 500  $\mu$ L of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) were added and incubated in the dark for 30 min at room temperature. The samples were centrifuged for 3 min at 20,000g, and the absorbance was read at 520 nm. TAC values were obtained by calculating the number of DPPH molecules scavenged per minute.

#### ***Determination of Catalase activity***

Catalase (CAT) activity was determined in psoas and soleus homogenate samples according to Aebi (1984) method (Aebi, 1984). 20  $\mu$ L of homogenate was added to 2975  $\mu$ L of sodium potassium phosphate buffer 67 mM, pH 7.4 and the samples were incubated at 37° C for 10 min. 5  $\mu$ L of hydrogen peroxide 30% was added and the change in absorbance was immediately read at 240nm for 2 min. One unit of catalase is equal to 1  $\mu$ mol of H<sub>2</sub>O<sub>2</sub> decomposed/minute. Results were normalized to total protein content in the homogenatesample (units/mg protein).

#### ***Spectrophotometric DNP assay for protein carbonyls content determination***

Protein carbonyls in oxidized proteins were determined in psoas and soleus homogenate samples according to Fields and Dixon (1971) (Fields & Dixon, 1971). In

50  $\mu\text{L}$  of plasma, 50  $\mu\text{L}$  of 20 % was added, incubated in the ice bath for 15 min and centrifuged at 15,000 X g for 5 min. at 4° C. the supernatant was discarded. Afterwards, 500  $\mu\text{L}$  of 14 mM 2,4-dinitrophenylhydrazine (DNPH) for the sample or 500  $\mu\text{L}$  of 2.5 N HCl for the blank was added to the pellet. Both samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min. Samples were centrifuged at 15,000 X g for 5 min. at 4° C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed, and centrifuged at 15,000g for 5 min at 4° C. The supernatant was discarded, and 1 mL of ethanol–ethyl acetate (1:1 v/v) was added, vortexed, and centrifuged at 15,000g for 5 min at 4° C. The washing step was repeated two more times. The supernatant was discarded, and 1 mL of 5 M urea (pH 2.3) was added, vortexed, and incubated at 37° C for 15 min. The samples were centrifuged at 15,000 X g for 3 min at 4° C, and the absorbance was read at 375 nm. Protein carbonyls values were obtained by using the molar extinction coefficient of 2,4-dinitrophenylhydrazine ( $22 \text{ mM}\cdot\text{cm}^{-1}$ ).

#### ***TBARS determination***

Thiobarbituric-acid reactive substances (TBARS) were determined in in psoas and soleus homogenate samples according to Buege and Aust (1978) (Buege & Aust, 1978). In 100  $\mu\text{L}$  of plasma, 500  $\mu\text{L}$  of 35 % TCA and 500  $\mu\text{L}$  of 200 mM Tris- HCl (pH 7.4) were added and incubated at room temperature for 10 min. Afterwards, 1 ml of 2 M  $\text{Na}_2\text{SO}_4$  and 55 mM thiobarbituric acid (TBA) solution was added and incubated at 95° C for 45 min. The samples were cooled on ice for 5 min and were vortexed. 1 ml of 70 % TCA was added, vortexed and centrifuged at 15,000 for 3 min at 25° C. The absorbance of the supernatant was read at 530nm. TBARS values were obtained using the molar extinction coefficient of MDA (15600 mol/l).

All materials for oxidative stress assays were purchased from Sigma (St. Louis, MO, USA)

#### **Statistical Analysis**

Duplicate values were averaged. Data were analyzed using the commercially available statistical software package SPSS 22. The Shapiro-Wilk test was performed to initially test whether the data were normally distributed, as it was the case. Results are expressed as mean  $\pm$  SEM and 95% confidence intervals.

Two-way MANOVA (two groups  $\times$  two muscles) was conducted to examine the effects of uremia and muscle type on muscle's redox status indices. Significant interactions and main effects were further investigated using LSD post-hoc analysis for multiple group comparisons. The significance level was set at  $p < 0.05$ .

## Results

Psoas and soleus muscle analysis results are summarized in Tables 4.2.1 and 4.2.2 respectively. [It should be noted that analysis there after refers to n=5 for Control group and n=8 for Uremic group as a batch of samples became inappropriate for analysis].

No interactions were found between the examined muscle types (psoas, soleus) and groups (Control, Uremic) for all the redox status indices ( $p > 0.05$ ). Analytically, Total Protein ( $F_{(1,21)}=2.857$ ,  $p=0.106$ ), PC ( $F_{(1,21)}=2.482$ ,  $p=0.130$ ), TBARS ( $F_{(1,21)}=0.270$ ,  $p=0.872$ ), GSH ( $F_{(1,21)}=0.761$ ,  $p=0.393$ ), GSSG ( $F_{(1,21)}=0.207$ ,  $p=0.654$ ), Ratio (GSH/GSSG) ( $F_{(1,21)}=0.921$ ,  $p=0.348$ ), GR-Reductase ( $F_{(1,21)}=0.957$ ,  $p=0.339$ ), TAC ( $F_{(1,21)}=0.020$ ,  $p=0.889$ ), CAT ( $F_{(1,21)}=0.229$ ,  $p=0.637$ ).

Significant group main effects were found for PC concentration ( $F_{(1,21)}=8.902$ ,  $p=0.007$ ) in both muscle types. The LSD post post-hoc test revealed that PC concentration was significantly higher in the Uremic group (psoas:  $1.086 \pm 0.294$ , soleus:  $2.52 \pm 0.29$  nmol/mg protein) compared to the Control (psoas:  $0.596 \pm 0.372$ , soleus:  $0.929 \pm 0.41$  nmol/mg protein). (Fig. 4.2.1). No significant group main effects were found in the rest of redox status indices ( $p > 0.05$ ).

Significant muscle main effects were found for Total protein concentration ( $F_{(1,21)}=23.166$ ,  $p=0.000$ ), PC concentration ( $F_{(1,21)}=6.410$ ,  $p=0.019$ ), TBARS concentration ( $F_{(1,21)}=14.703$ ,  $p=0.001$ ) (Fig.1), GSH concentration ( $F_{(1,21)}=6.175$ ,  $p=0.021$ ), TAC concentration ( $F_{(1,21)}=18.316$ ,  $p=0.000$ ), Catalase activity ( $F_{(1,21)}=20.597$ ,  $p=0.000$ ) and GR-Reductase activity ( $F_{(1,21)}=7.498$ ,  $p=0.012$ ) (Fig.2) in both Control and Uremic group, respectively. The LSD post post-hoc test revealed that Total protein and TAC concentrations were significantly lower in soleus compared to psoas muscle in both the Control and the Uremic group. Additionally, soleus demonstrated higher levels of TBARS and PC levels as well as higher GSH levels, Catalase and GR-Reductase activities compared to psoas muscle in both the Control and Uremic group. The results are presents in Figures 4.2.1 & 4.2.2.

**Table 4.2.1.** Psoas biochemical and redox status indices (in Mean  $\pm$  SEM) in the Control and Uremic group. The 95% Confidence Intervals are reported.

PSOAS MUSCLE	CONTROL GROUP (n=5)	95% Confidence Interval		UREMIC GROUP (n=8)	95% Confidence Interval	
		Lower Bound	Upper Bound		Lower Bound	Upper Bound
Total Protein (mg/ml)	5.753 $\pm$ 0.43	4.865	6.642	4.537 $\pm$ 0.34	3.836	5.240
PC (nmol/mg protein)	0.596 $\pm$ 0.372	0.179	1.370	1.086 $\pm$ 0.294*	0.474	1.699
TBARS (nmol/ml)	3.179 $\pm$ 1.06	0.983	5.376	2.297 $\pm$ 0.835	0.561	4.034
GSH ( $\mu$ mol/g protein)	5.539 $\pm$ 1.69	2.014	9.065	6.087 $\pm$ 1.34	3.300	8.875
GSSG ( $\mu$ mol/g protein)	0.265 $\pm$ 0.09	0.060	0.470	0.322 $\pm$ 0.07	0.160	0.484
Ratio (GSH/GSSG)	18.66 $\pm$ 11.11	4.455	41.775	25.60 $\pm$ 8.787	7.326	43.874
GR Reductase (U/g protein)	10,59 $\pm$ 2.25	5.912	15.273	12.72 $\pm$ 1.78	9.018	16.419
TAC ( $\mu$ mol DPPH/ml)	0.595 $\pm$ 0.06	0.458	0.732	0.519 $\pm$ 0.05	0.411	0.627
CAT (U/mg protein)	6.992 $\pm$ 4.71	2.799	16.785	5.721 $\pm$ 3.72	-2.020	13.463

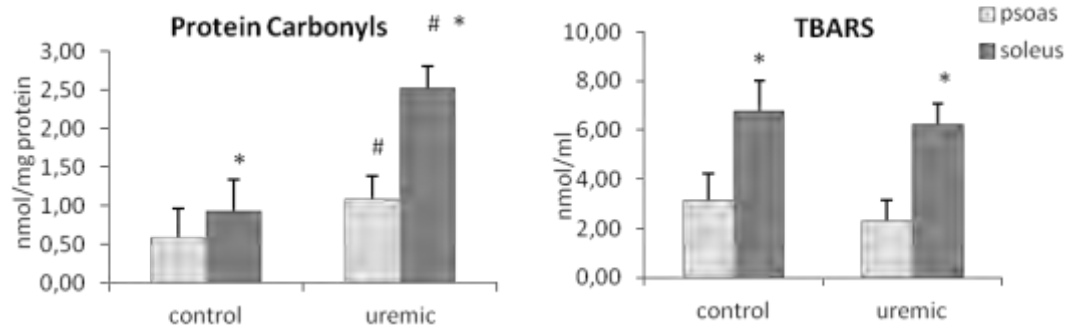
PC: Protein Carbonyls, TBARS: Thiobarbituric Acid Reactive Substances, GSH: Reduced Glutathione, GSSG: Glutathione Oxidized, TAC: Total Antioxidant Capacity, CAT: Catalase, \* statistical significance between Control and Uremic group,  $p < 0.05$ .



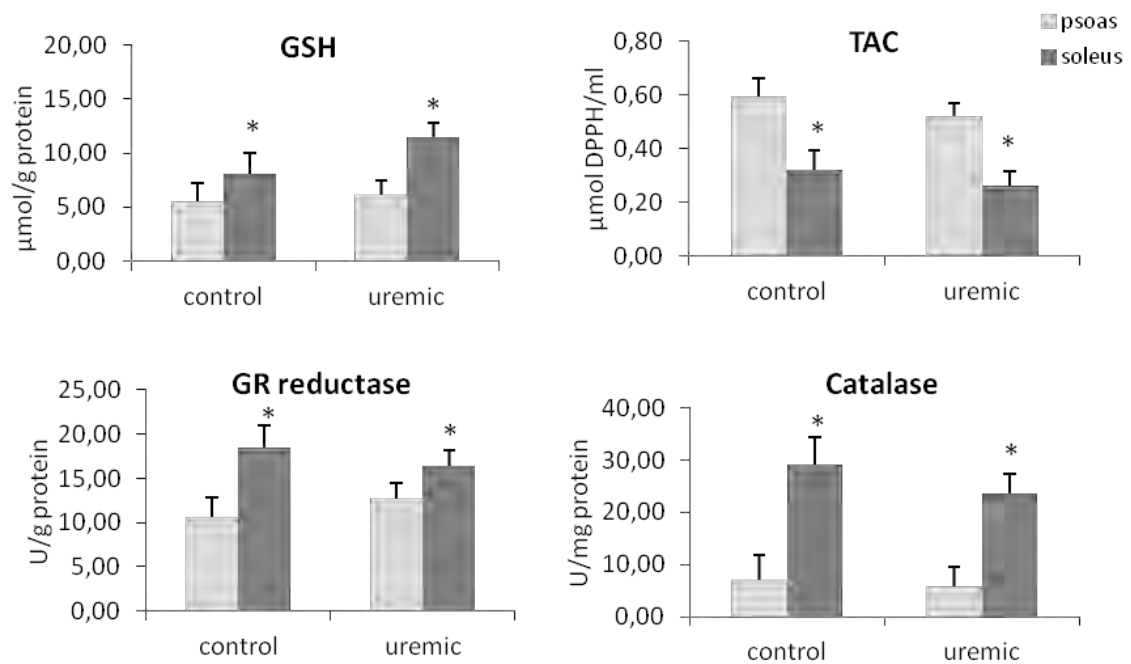
**Table 4.2.2.** Soleus biochemical and redox status indices (Mean  $\pm$  SEM) in the Control and Uremic group. The 95% Confidence Intervals are reported.

SOLEUS MUSCLE	CONTROL GROUP (n=5)	95% Confidence Interval		UREMIC GROUP (n=8)	95% Confidence Interval	
		Lower Bound	Upper Bound		Lower Bound	Upper Bound
Total Protein (mg/ml)	3.155 $\pm$ 0.478	2.161	4.148	3.29 $\pm$ 0.33	2.588	3.992
PC (nmol/mg protein)	0.929 $\pm$ 0.41	0.063	1.795	2.52 $\pm$ 0.29*	1.905	3.129
TBARS (nmol/ml)	6.81 $\pm$ 1.18	4.350	9.262	6.25 $\pm$ 0.83	4.511	7.984
GSH ( $\mu$ mol/g protein)	8.09 $\pm$ 1.89	4.155	12.039	11.41 $\pm$ 1.34	8.625	14.20
GSSG ( $\mu$ mol/g protein)	0.331 $\pm$ 0.11	0.102	0.560	0.472 $\pm$ 0.78	0.310	0.634
Ratio (GSH/GSSG)	46.62 $\pm$ 12.43	20.782	72.468	33.61 $\pm$ 8.79	15.34	51.89
GR Reductase (U/g protein)	18.41 $\pm$ 2.52	13.184	23.651	16.42 $\pm$ 1.78	12.724	20.125
TAC ( $\mu$ mol DPPH/ml)	0.322 $\pm$ 0.07	0.169	0.476	0.264 $\pm$ 0.05	0.155	0.372
CAT (U/mg protein)	29.09 $\pm$ 5.26	18.140	40.063	23.60 $\pm$ 3.72	15.863	31.346

PC: Protein Carbonyls, TBARS: Thiobarbituric Acid Reactive Substances, GSH: Reduced Glutathione, GSSG: Glutathione Oxidized, TAC: Total Antioxidant Capacity, CAT: Catalase, \* statistical significance between Control and Uremic group,  $p < 0.05$ .



**Fig. 4.2.1.** Protein Carbonyls (PC), (A) and TBARS, (B) concentration in psoas and soleus muscles in Control and Uremic groups. Concentrations are expressed as nmol/mg protein and nmol/ml respectively. \* depicts significant differences between psoas and soleus muscle, # depicts significant differences between Control and Uremic groups,  $p < 0.05$ .



**Fig. 4.2.2.** GSH and TAC concentrations (A, B), GR Reductase and Catalase activities (C, D) in psoas and soleus muscles in Control and Uremic groups. Concentrations and activities are expressed as μmol/g protein, μmol DPPH/ml, U/g protein, U/mg protein for GSH, TAC, GR reductase and Catalase respectively. \* depicts significant difference between psoas and soleus muscle,  $p < 0.05$ .

## Discussion

Apart from the more recognized systemic negative manifestations, CKD is also accompanied by muscular weakness and wasting, reduced endurance and premature fatigue, characteristics and symptoms which collectively described as uremic myopathy (Campistol, 2002). Several studies have indicated that CKD is characterized by modifications of cell energy metabolism leading to a reduction in muscle performance (Brautbar, 1983; Del Canale et al., 1986; Metcalf et al., 1983). It has been suggested that hemodialysis treatment and/or uremia per se contribute to an increase in molecular oxidative damage which in turn play a key role for loss of skeletal muscle functionality. Additionally, animal studies have shown that experimentally induced oxidative stress causes myofibrillar protein modification and degradation (Nagasawa et al., 1997) through alterations of its amino acid sequences. A variety of amino acid modifications exists with carbonyl formation to be considered as an early marker of protein oxidation. Arginine, proline and threonine are the most likely amino acids to form carbonyl derivatives (Reznick & Packer, 1994). Given that skeletal muscle contains high levels of fibrillar proteins, it becomes extremely susceptible to free radical oxidation with adverse functional outcomes.

The increased PC levels by approximately 1.82 fold for psoas and 2.71 fold for soleus in Uremic compared to Control samples, point to increased levels of sarcomeric protein carbonylation. Our findings are in agreement with those reported by Lim et al (Lim, Cheng, et al., 2002) in skeletal muscle of uremic patients on HD, which triggers an increased protein degradation. The increased levels of PC in pre-dialysis stage of CKD, as it was observed in our study, could be regarded as a very interesting finding for the time point of protein oxidation, revealing the early emergence of oxidative damage to protein content. Taking into account the role of carbonyl stress in vascular damage (Chen et al., 2013) and the general functionality of muscle, the early countermeasure for protein oxidation could be of great importance for patients before they move into the end-stage where the negative effects on muscle status may become irreversible.

No other redox index differed between the control and uremic groups. However we observed large differences between the two muscle types (psoas and soleus) studied.

Skeletal muscle is a heterogeneous tissue which consists of a large diversity of fiber types. This heterogeneity in muscle type composition determines distinct biochemical and structural characteristics which in turn are responsible for the variety of physiological and metabolic functions (Essen-Gustavsson, Karlström, & Lundström, 1992; Pette & Staron, 1990). In our study we examined the redox status of two different types of skeletal muscle, the slow twitch soleus (Fiber type I) and the fast twitch psoas (Fiber type II) in order to observe their possible different response in the uremic environment.

Total protein concentration was found increased in psoas muscle compared to soleus in both Control and Uremic group. It is known that skeletal muscle wasting is a characteristic of several chronic diseases (Espot, Copeland, & Moldawer, 1994) and is strongly related with loss of protein. One of the several suggested mechanisms is the tumor necrosis a (TNF- $\alpha$ ) – induced catabolism (Ahmad, Karlstad, Choudhry, & Sayeed, 1994; Buck & Chojkier, 1996). TNF- $\alpha$  can indirectly affect muscle catabolism via the regulation of hormones and catabolic cytokines (Dayer, Beutler, & Cerami, 1985; Gelin et al., 1991; Tessitore, Costelli, & Baccino, 1993), but can also promote muscle waste stimulating the ubiquitin gene expression (Llovera, Garcia-Martinez, Agell, Lopez-Soriano, & Argiles, 1997) and activating the nuclear factor NF- $\kappa$ B (Beg, Finco, Nantermet, & Baldwin, 1993). Li et al (Y. P. Li, Schwartz, Waddell, Holloway, & Reid, 1998), observed that TNF- $\alpha$  can directly trigger protein loss and decrease in myosin heavy chain (MHCf) levels in skeletal muscle. It is also known that TNF- $\alpha$ /NF- $\kappa$ B signaling is widely affected from endogenous ROS. TNF- $\alpha$  widely excites mitochondria ROS production, promoting TNF- $\alpha$ /NF- $\kappa$ B activation (Hennet, Richter, & Peterhans, 1993; Schulze-Osthoff et al., 1992), a process which seems to be tissue specific (Y. P. Li et al., 1998). Taking into consideration that soleus muscle (type I fibers) contains a higher amount of mitochondria, it would be rather logical that it would be more sensitive to NF- $\kappa$ B activation and therefore, to protein loss and muscle wasting. Our findings, where soleus muscle exhibited lower total protein levels, seem to confirm this observation.

Regarding the oxidative damage that was examined in two muscles, protein carbonylation and lipid peroxidation as were expressed via PC and TBARS levels respectively, it was significantly increased in soleus muscle compared to psoas in both Control group and Uremic group. Specifically, levels of TBARS were higher in the soleus than psoas muscles without a differentiation between the control and uremic groups, (2.14 fold for control and 2.72 fold for uremic). We have not seen analogous data in pre-dialysis patients. In HD patients however, Lim et al reported increased TBARS levels in skeletal muscle (sampled during fistula creation) (Lim, Cheng, et al., 2002) compared to healthy controls. It is known that in these patients extensive oxidation damage is usually observed, with this type of therapy to constitute one more aggravating factor.

Slow-twitch skeletal muscles such as soleus, have a greater number of mitochondria (Engel, 1970), which are regarded to play a key role in the ROS production in skeletal muscle, due to their increased oxygen consumption (McArdle, Pattwell, Vasilaki, Griffiths, & Jackson, 2001). Based on the increased amount of mitochondria in soleus muscle, it could be expected that this type of skeletal muscle by increasing ROS production it may be more exposed to ROS and their negative effects, but also that it develops an appropriate antioxidant capacity to withstand the oxidative load. Despite the reduced TAC levels in soleus muscle in comparison with psoas, the increased GSH, CAT and CR-reductase indicates that healthy soleus indeed demonstrates a higher antioxidant capacity than psoas.

In response to a further oxidative load due to renal insufficiency, uremic soleus appears to have further upregulated its defenses. Thus, we observed a further increased GSH levels and GR-Reductase and CAT activities in uremic soleus muscle compared to uremic psoas. As it is known, GR- Reductase is an enzyme which catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form GSH. These two antioxidant molecules together with CAT activity constitute the antioxidant defense which has been brought into action as an adaptive response against the increased oxidative damage in soleus muscle.

## Conclusion

The increased PC levels in soleus in Uremic compared to Control samples, point to increased levels of sarcomeric protein carbonylation. Since carbonyl formation is associated with protein degradation and proteolysis, the increased levels seem to constitute an early stimulus to muscle atrophy observed in the early stages of CKD development. Thus early countermeasures for protein oxidation could be of great importance for patients before they move into the end-stage where the negative effects on muscle status may become irreversible

Both oxidative damage and antioxidant response seem to be tissue-specific. Soleus muscle, which has greater number of mitochondria, may be more exposed to ROS and their negative effects, but also may develop an appropriate antioxidant capacity to withstand the oxidative load. This was reflected in the measured GSH values in soleus muscles. In response to a further oxidative load due to renal insufficiency, uremic soleus appears to have further upregulated its defenses in comparison with uremic psoas.

Our results highlight the need for interventions early during disease progression in order to protect skeletal muscle quality and reach the end-stage at the best possible functional and biochemical status. How exactly CKD accelerates protein carbonylation and degradation remains to be determined in future work.

**Research paper 3: CKD\_ Hematological and Biochemical changes  
in pre-dialysis stage of the disease**

## Abstract

**Introduction:** Chronic Kidney Disease (CKD) constitutes a major health problem worldwide and is accompanied by several comorbidities such as anemia, cardiovascular diseases, atherosclerosis, diabetes, which contribute to the reduced quality of life and to the mortality of the patients.

**Aims:** This aim conducted to evaluate the biochemical and hematological parameters using an animal model of renal insufficiency where confounding factors such as comorbidities and pharmacological treatment are not implicated.

**Methods:** We used an animal model (partial nephrectomy) of renal disease in New Zealand white female rabbits. Surgery and euthanasia (after 3 months) protocols were approved by the ethic committee of the University of Thessaly. Renal insufficiency was induced by removal of the right kidney and partial nephrectomy of the left kidney (Uremic group, N=9). Control animals underwent sham operation (Control group, N=6). Blood samples were collected and analysed for the biochemical (lipid profile) indices Total Cholesterol, High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Triglycerides, Triglycerides/HDL Ratio Glucose and Bilirubin and for the hematological parameters leukocytes, red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH).

**Results:** We found no significant changes in the blood lipid profile of the Uremic group as compared to the Control group ( $p < 0.05$ ). However, the Ratio Triglycerides/HDL was 5.67, much higher than 4. Moreover, RBC, Hematocrit and MCH were found significant decrease in the Uremic group ( $3.92 \pm 0.29$ ,  $26.06 \pm 1.85$ ,  $19.76 \pm 0.67$ ) as compared to the Control ( $5.10 \pm 0.17$ ,  $35.24 \pm 0.79$ ,  $21.64 \pm 0.44$ ),  $p < 0.05$ , and MCV had a tendency to decrease in the Uremic ( $65.40 \pm 1.89$ ) group compared to the Control group ( $69.78 \pm 1.36$ )  $p = 0.087$ .

**Conclusion:** The lipid profile of our Uremic group did not seem to be disturbed in a great extent. A tendency however, for an increased risk for coronary disease emerged through the calculation of the Triglycerides/HDL atherogenic index. Moreover significant signs of anemia manifestation were found suggesting that early



development of anemia, in pre dialysis stage, could be linked to muscle mechanisms of fatigue by the reduction of oxygen carrying capacity.

## Introduction

Chronic Kidney Disease (CKD) constitutes a major health problem worldwide (Hsu, Bates, Kuperman, & Curhan, 2001) and is accompanied by several comorbidities which contribute to the increased mortality of the patients.

Cardiovascular complications are a major cause of mortality in patients with end-stage renal failure (K. Ma, Greene, & Rajj, 1992). In these patients, several common atherogenic risk factors, such as hypertension, diabetes, dyslipidemia (Attman & Alaupovic, 1991; K. Ma et al., 1992) are combined with factors related to the uremic state, such as increased serum levels of homocysteine (Bostom et al., 1997), and lipoprotein (Sechi et al., 1998) with adverse outcomes. Some of these risk factors could be detected early in the course of CKD (Jensen, Stender, & Deckert, 1988) and may contribute to the development of cardiovascular complications in CKD patients. It has been suggested that often CKD patients are more likely to die from cardiovascular complications before full kidney failure development (Shulman et al., 1989). The increased CVD events in CKD are partly attributed to accelerated atherogenesis caused by dyslipidemia (London & Drueke, 1997). The common lipid abnormalities in pre-dialysis patients include high-density lipoprotein (HDL) and high triglyceridemia while low-density lipoprotein (LDL) and total cholesterol (TC) may be high, low or normal (Agaba IE, Agbaji OO, Anteyi EA, Omudu PA, & RS., 2003; Ojo OE, Soyinka FA, Sanusi AA, Arogundade FA, & A., 2007).

Additionally, it has been reported that dyslipidemia plays a key role to glomerular and tubulo-interstitial injuries (Keane, Mulcahy, Kasiske, Kim, & O'Donnell, 1991; Moorhead, 1991; Moorhead, Chan, El-Nahas, & Varghese, 1982) and thus contributes to the progression of renal insufficiency per se. Moreover, organ fat infiltration has been reported in end-stage patients. This is not only confined to the liver but is present in major limb skeletal muscles (Giannaki et al., 2013), and is associated with sleep problems and the presence of metabolic syndrome (Sakkas, Gourgoulianis, et al., 2008). Not surprisingly, disorders of glucose homeostasis affect approximately 50% of patients suffering from CKD. Glucose homeostasis depend on sufficient production of insulin from pancreatic  $\beta$ -cells and adequate insulin action on peripheral tissues. Thus glucose handling is carefully monitored upon CKD diagnosis.

Anemia is also a common clinical manifestation in patients with CKD and it is associated with fatigue, reduced exercise capacity, cognitive impairment and increased risk of cardiovascular disease and hospitalization, resulting in reduced quality of life and mortality (Silverberg, Wexler, Blum, Wollman, & Iaina, 2003). Anemia has been defined by the World Health Organization (WHO) as a hemoglobin (Hb) concentration < 13.0 g/dl for adult males and postmenopausal women and an Hb<12.0 g/dl for premenopausal women ("World Health Organization.Nutritional Anaemias: Report of a WHO Scientific Group.," 1968). Based upon these criteria, nearly 90% of patients with GFR<25-30 ml/min (stages 4 and 5) have anemia, many with Hb<10g/dl (Kazmi et al., 2001). Although the exact mechanisms have not been fully elucidated, several metabolic and mechanical factors have been proposed (J. W. Eschbach, Jr. et al., 1967). Shortened red blood cell life and increased red cell apoptosis, with the hemolytic syndrome playing an important role, and decreased red cell production in bone marrow constitute the most common etiologies for anemia in CKD (Erslev & Besarab, 1997). Erythropoietin (EPO) deficiency is one of the predominant causes of anemia in CKD. It has been also suggested that circulating uremic toxins behave as inhibitors for the erythropoiesis process and/or the responsiveness to the hormone and contribute to anemia but no study has so far managed to observe and identify specific inhibitors pathways (Besarab & Ayyoub, 2007b; J. W. Eschbach, 1989).

Although there has been an improvement in prognosis for CKD patients over the past decade there are yet a lot to be done. An early detection of discrepancies in hematological and biochemical parameters may prove significant since treating these abnormalities may prevent the progression of renal insufficiency and delay adverse outcomes of CKD, including anemia and atherosclerosis. However, as alluded above, various comorbidities can influence hematological and muscular levels.

This study was conducted to evaluate the biochemical and the hematological parameters using an animal model that correspond to an advanced but in pre-dialysis stage of CKD where confounding factors such as comorbidities and pharmacological treatment are not implicated. Thus, possible early findings could suggest new directions on how to use diagnostic and therapeutic procedures, which upon verification on human patients could help to improve care, patient's functional condition, quality of life and probably the risk of morbidity and mortality.

## Methods

### Animals and experimental design

New Zealand white female rabbits (young adult, N=15) with body weight of approximately 3200 kg, were first acclimatized to the laboratory animal unit of Medical School (University of Thessaly, Greece) for 48 hours. The animals were housed in a controlled environment with stable conditions of room temperature (RT) (22–24°C) and lighting (12:12 h light-dark cycle). All rabbits were fed with a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum. All animal procedures, including surgery and euthanasia for this project were approved by the ethics committee of the University of Thessaly (decision 2-2/10-10-2012) and the scientific committee of the University Hospital of Larisa, Greece (decision 1/4-1-2012) and animals were under veterinary care, in accordance to the national directives for the care and the use of laboratory animals.

After acclimatization, surgical procedures were performed (sham operation for control animals - Control group and partial nephrectomy for experimental animals – Uremic group. Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87 % and 13 % respectively (proportion 6,69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg body weight of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anesthesia was achieved by a dose of propofol (10mg/kg BW). Three hours before the intervention, each animal had only access to water and not to food and its weight was measured on a precision scale. Animal temperature was maintained via a heating pad.

The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982 (Gotloib et al., 1982). For the Uremic group nine animals (N=9), underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy ( $\frac{3}{4}$ ) of the right kidney. For the Control group six age-matched animals (N=6) underwent sham operation. Twelve weeks after surgery, the animals were weighed and then sacrificed by injection of sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100

mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, sample collections were done in a blind fashion.

### **Sample preparation and Biochemical Analyses**

#### ***Blood sampling and treatment***

Whole blood was collected from the rabbits' heart and aorta by a heparinized syringe and placed into evacuated test tubes. A portion (1-2 ml) of the blood collected was used to determine leukocytes, red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) using a Mythic 18 (Orph e S.A., Geneva, Switzerland) autoanalyzer.

For serum collection, another portion of blood (5 ml) blood was placed into separate tubes containing clot activator (Vacutainer, BD, Franklin Lakes, NJ, USA) and was allowed to clot for 20 min at room temperature. Afterwards, tubes were centrifuged at  $1370 \times g$  for 10 min at  $4^{\circ} C$  and The supernatant was collected, aliquoted in eppendorf tubes, stored at  $-80^{\circ}C$  and thawed only once before analysis. Glucose, Bilirubin , Total Cholesterol, High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL) and Triglycerides concentrations were measured on a Clinical Chemistry Analyzer Z 1145 (Zafiroopoulos Diagnostica, Athens, Greece) using commercially available kits (Zafiroopoulos Diagnostica).

#### **Statistical Analysis**

Duplicate values were averaged. Data were analyzed using the commercially available statistical software package SPSS 22. The Shapiro-Wilk test was performed to initially test whether the data were normally distributed, as it was the case. Results are expressed as mean  $\pm$  SEM and 95% confidence intervals.

An independent t-test was conducted to examine whether there were any differences in blood redox status indices between Control and Uremic group. The significance level was set at  $p < 0.05$ .

## Results

### *Blood biochemical analysis*

No significant differences were found in the concentrations of Total Cholesterol, HDL, LDL, Triglycerides and Glucose levels in blood of Control and Uremic groups ( $p>0.05$ ), as presented in Table 4.3.1. Bilirubin concentration tended to be higher in the Uremic group compared to Control ( $p=0.074$ ).

**Table 4.3.1.** Blood biochemical indices (in Mean  $\pm$  SEM) in the Control and Uremic group. The exact statistical significance value P and the 95% Confidence Intervals are reported

BLOOD	CONTROL GROUP (n=6)	95% Confidence Interval		UREMIC GROUP (n=9)	95% Confidence Interval		P
		Lower Bound	Upper Bound		Lower Bound	Upper Bound	
Total Cholesterol (mg/dl)	102.73 $\pm$ 13.59	91.86	113.61	95.16 $\pm$ 11.94	71.75	118.57	0.683
HDL (mg/dl)	29.77 $\pm$ 6.12	24.87	34.66	27.36 $\pm$ 4.49	18.55	36.17	0.758
LDL (mg/dl)	51.07 $\pm$ 13.71	40.10	62.05	50.31 $\pm$ 13.64	23.57	77.05	0.969
Triglycerides (mg/dl)	109.46 $\pm$ 17.45	95.49	123.43	121.05 $\pm$ 14.92	91.80	150.30	0.623
Triglycerides/HDL Ratio	4.63 $\pm$ 1.33	3.56	5.70	5.97 $\pm$ 1.25	3.66	8.27	0.545
Glucose (mg/dl)	388.25 $\pm$ 37.16	358.352	417.98	311.89 $\pm$ 31.48	250.19	373.58	0.145
Bilirubin (mg/dl)	0.247 $\pm$ 0.03	0.224	0.273	0.418 $\pm$ 0.08	0.263	0.575	0.074

Significant level at ( $P < 0.05$ )\* HDL: High-Density Lipoprotein, LDL: Low-Density Lipoprotein

### *Hematological analysis*

The Hematocrit levels were found to be decreased in the Uremic group compared to Control, ( $t(11)= 3.732$ ,  $p=0.001$ ). Red blood cell count was significantly decreased in Uremic group compared to Control, ( $t(11)= 3.014$ ,  $p=0.012$ ). Mean Corpuscular Hemoglobin was found decreased in Uremic group compared to Control, which was statistically significant  $t(11)= 2.333$ ,  $p=0.040$ .

The leukocyte count was not statistically different from control ( $p>0.05$ ). Likewise Hemoglobin concentration tended to be lower in the Uremic group compared to Control, which is not statistically significant ( $p>0.05$ ). Mean Corpuscular Volume was found decreased in uremic group, which was not statistically significant ( $p>0.05$ ). All data were presented in table 4.3.2.

**Table 4.3.2.** Hematological indices (in Mean  $\pm$  SEM) in the Control and Uremic group. The exact statistical significance value P and the 95% Confidence Intervals are reported.

BLOOD	CONTROL GROUP (n=6)	95% Confidence Interval		UREMIC GROUP (n=9)	95% Confidence Interval		P
		Lower Bound	Upper Bound		Lower Bound	Upper Bound	
Leukocyte count ( $\times 10^3/\mu\text{L}$ )	11.12 $\pm$ 1.76	9.58	12.66	10.86 $\pm$ 1.02	8.86	12.87	0.903
Red Blood Cells ( $\times 10^6/\text{mm}^3$ )	5.10 $\pm$ 0.17	4.95	5.25	3.92 $\pm$ 0.29	3.56	4.48	0.012*
Hemoglobin (mg/ml)	110.77 $\pm$ 9.20	103.36	118.17	97.99 $\pm$ 10.08	78.22	117.76	0.368
Hematocrit (%)	35.24 $\pm$ 0.79	35.54	35.93	26.06 $\pm$ 1.85	22.43	29.69	0.003*
MCV ( $\text{mm}^3$ )	69.78 $\pm$ 1.36	68.59	70.97	65.40 $\pm$ 1.89	61.68	69.12	0.087
MCH ( $\mu\text{g}/\text{cell}$ )	21.64 $\pm$ 0.44	21.26	22.02	19.76 $\pm$ 0.67	18.44	21.09	0.04*

Significant level at (P < 0.05)\* MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin

## Discussion

Our study examined a range of biochemical and hematological indices in an animal model of chronic renal insufficiency. While there were no significant changes in the blood lipid and glucose profile of the Uremic group as compared to the Control group, we observed a consistent decline in hematological parameters related to anemia and overall fitness status.

We examined whether dyslipidemia could be traced in an animal model of pre-dialysis CKD and compared the findings with those of the Control group (sham-operated). We found no significant differences in Total Cholesterol, HDL, LDL or Triglycerides levels between the two groups. However we observed that Triglycerides/HDL ratio was  $> 4$  in both groups but in uremic group it was 1.3 fold higher. Perhaps, if the duration of our model was longer a more substantial change in the lipid profile could have occurred.

There is strong epidemiological and clinical evidence that increase in plasma LDL and decrease in plasma HDL concentrations are associated with increased atherosclerotic complications (Jungers et al., 1997; London & Drueke, 1997) partly attributed to the synergistic action of both LDL and HDL. While increased LDL levels are associated with increased deposition in the vessel wall, low HDL levels decrease the reverse cholesterol transport from tissues to the liver leading to their accumulation in the tissues. Akpan et al., 2014 (Akpan, Ekrikpo, Effa, Udo, & Kadiri, 2014) evaluated lipid abnormalities in pre-dialysis patients with CKD from stage 1-5. The study revealed that total cholesterol (TC) and LDL were elevated above normal levels compared to controls, (Akpan et al., 2014).

We did not find statistically significant differences in Cholesterol fractions between the Uremic and Control groups. Species and analysis methods aside, perhaps more time was needed for our model of uremia to develop such changes in Cholesterol. Furthermore, diet composition is known to influence blood biochemical parameters. A change in lipid blood parameters in a short period of time could be due to a change of diet lipid composition. The rabbits of the present study however were fed the same diet (3.5% fat content), our results could not be attributed to a change of diet.



The ratio of Triglycerides/HDL, initially proposed by Gaziano et al (Gaziano, Hennekens, O'Donnell, Breslow, & Buring, 1997) has been proposed as an atherogenic index that has proven to be a highly significant independent predictor of myocardial infarction, even stronger than Total Cholesterol/HDL and LDL/HDL. Jeppesen et al (Jeppesen, Hein, Suadicani, & Gyntelberg, 1998) also found that stratifying triglycerides levels by HDL levels led to more accurate detection of increased risk of coronary disease. The atherogenic link between high triglycerides and HDL-c is due to the higher plasma concentration of triglyceride-rich, very low-density lipoprotein that generates small, dense LDL during lipid exchange and lipolysis. More specifically, the ratio of Triglycerides/HDL correlates inversely with the plasma level of small, dense LDL particles. These LDL particles accumulate in the circulation and form small, dense HDL particles, which undergo accelerated catabolism, thus closing the atherogenic circle (Brinton, Eisenberg, & Breslow, 1991; Edwards, Mahaney, Motulsky, & Austin, 1999). Regarding the ratio value, da Luz et al (da Luz, Cesena, Favarato, & Cerqueira, 2005) found that a Triglycerides/HDL ratio  $> 4$  constitutes the most powerful independent predictor of coronary artery disease and another the study of the same group (da Luz, Favarato, Faria-Neto, Lemos, & Chagas, 2008) indicated that a ratio of Triglycerides/HDL  $> 4$  is also related to the severity of vessel compromise being an easy, non-invasive method of predicting the presence and extent of coronary atherosclerosis.

Additionally, in a previous study (Research Paper 1) we observed a tendency for increased lipid peroxidation (TBARS) in the plasma of the Uremic compared to the Control. Thus, the tendency for a higher Triglycerides/HDL and increased levels of lipid peroxidation in the Uremic group fits well with a progressive atherogenic development due to chronic renal insufficiency.

There were significant decreased in the levels of hematological parameters (RBC, Hematocrit, MCH) in the Uremic group as compared to the Control. RBC count was found to be decreased in the Uremic group. The mean corpuscular hemoglobin or mean cell hemoglobin (MCH) was significantly lower in the Uremic group. MCH reflects the average mass of hemoglobin per red blood cell, and lower values may be linked to hypochromic anemias and microcytic RBC. The latter is possible as we observed a tendency for lower mean corpuscular volume or mean cell volume (MCV), in the Uremic group. MCV reflects the average volume of a red

blood cell and is also associated with several types of anemia. However, both MCH and MCV values in Uremic animals were at the lower ends of the physiological range.

The above hematological disturbances could be explained by an impairment in the erythropoietin production and other factors which influence negatively the red cell production in bone marrow and the red cell lifespan. Indeed, the expression of phosphatidylserine, an important phospholipid membrane component which plays a key role in cell cycle signaling and apoptosis, is found to be increased in the plasma of uremic patients. As a result, the damaged red blood cells are better recognized and removed by phagocytes (Fadok, Bratton, Frasch, Warner, & Henson, 1998; Jeng & Glader, 2004). A higher apoptotic rate could be hypothesized in our model from the higher bilirubin levels observed in the experimental group.

Overall, taking into consideration the fact that Hb, Hct and MCH levels and red blood cell count in the Uremic group were at the lower limits of rabbit physiological values it is concluded that these findings constitute early signs of anemia development.

The degree of anemia in CKD has been explored by several researchers and different ways of treatment has been proposed. Human studies have evaluated the prevalence of anemia in patients in different stages of CKD. Kazmi et al., observed that the prevalence of anemia for Hb<12 g/dl (the level at which the evaluation of anemia in CKD should be initiated) was 45 %, 49%, 58%, 92%, 92% in CKD stages 1-5 respectively and for Hb<11g/dl (the minimum hemoglobin levels at which therapy should be initiated with EPO) as 24%, 34%, 41%, 79%, 74% respectively for stages 1-5 (Kazmi et al., 2001). Similarly Shaheen et al., found that the prevalence of anemia was elevated for Hb<12g/dl in the different stages of CKD (Shaheen et al., 2011). The recombinant human erythropoietin, epoetin alfa (rHuEpo) was the first Erythropoiesis-Stimulating Agent (ESA) for the treatment of anemia reducing the transfusions and their side-effects such as infections and iron overload (Popovsky & Ransil, 1996). Several concerns (Garcia, Anderson, Rennke, & Brenner, 1988) about possible impairment of kidney function due to EPO treatment, have been rejected by clinical evidence (Kuriyama et al., 1997; Roth et al., 1994; Teplan et al., 2003). However, the administration of EPO in pre-dialysis stage patients with CKD is not so much popular since anemia development is not so clear.

Although the United States guidelines recommend EPO treatment when hemoglobin drops below 10 g/dl and the European guidelines recommend it for hemoglobin levels below 11 g/dl ("KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease,"), EPO administration is not common in clinical practice (Horl et al., 2003; Valderrabano et al., 2003) among patients and most of them start EPO treatment when anemia is well advanced. This occurs despite findings on benefits of early EPO administration. For example, Gouva et al (Gouva, Nikolopoulos, Ioannidis, & Siamopoulos, 2004) observed that early initiation of EPO treatment in pre-dialysis patients with non-severe anemia is extremely beneficial resulting in 60% reduction in the risk of initiation of kidney replacement or death. Also, several studies highlighted the benefits of correcting anemia such as improved exercise capacity, cognitive function and improvement of left ventricular performance (Clyne & Jogestrand, 1992; Hayashi et al., 2000; Portoles et al., 1997).

Our findings of low hematocrit and overall disturbed hematological parameters in an animal model of pre-dialysis CKD further verify that our model represents an (untreated) human renal disease condition (when EPO is not supplemented). Taken together, our observations and the human data, these findings indicate that anemia could have a major role in disease development and symptoms severity and should be addressed as early as possible.

## **Conclusions**

Our model of CKD shows that the Lipid profile in this stage of the disease development does not seem to be disturbed to a great extent. However, an atherogenic development cannot be excluded and should be addressed early on.

More importantly, significant signs of hematological disturbances were found suggesting that early recognition of anemia development in the pre-dialysis stage could play a key role in CKD treatment.

**Research paper 4: Do blood redox status indices reflect changes in skeletal muscle's redox status?**

## Abstract

**Introduction:** The redox status of an individual under normal or pathological conditions is assessed by evaluating a group of indices in non-invasive samples. However there is no information about tissue localization of oxidative stress. The large majority of studies examining CKD and discussing comorbidities, such as uremic myopathy, have reported increased levels of oxidative stress by only assessing indices in blood samples of CKD patients without clear information of what is happening in the redox status of skeletal muscle

**Aims:** The aim of the study was to examine whether the oxidative stress markers measured in blood adequately reflects redox status in skeletal muscle. Moreover it was examined if blood urea and creatinine levels in blood had any possible reflection on blood and skeletal muscle redox status and if there is a correlation between urea and creatinine levels. Finally it was investigated whether redox status in psoas muscle adequately depicts the redox status in soleus. All correlation analyses were conducted in pool data, as well as in Control group and in the Uremic group separately.

**Methods:** We used an animal model (partial nephrectomy) of renal disease in New Zealand white female rabbits. Surgery and euthanasia (after 3 months) protocols were approved by the ethic committee of the University of Thessaly. Renal insufficiency was induced by removal of the right kidney and partial nephrectomy of the left kidney (Uremic group, N=9). Control animals underwent sham operation (Control group, N=6). Blood and skeletal muscle (psoas, soleus) samples were collected and analysed for the GSH, GSSG, GSH/GSSG ratio, GR-Reductase, CAT, TAC, TBARS and PC levels.

**Results:** For each one of the redox status indices, statistical analysis didn't show any significant correlation between blood and the two examined muscles' levels neither in the pool of samples nor for each group separately ( $p > 0.05$ ). Regarding the correlation analysis between Urea, Creatinine and redox status in blood and skeletal muscle, while no relationship reached statistical significance it is noteworthy that in the pool data Creatinine levels tended to correlate with blood GSH ( $r=0.664$ ,  $p=0.073$ ). Likewise, Urea tended to correlate with blood GSH ( $r=0.655$ ,  $p=0.078$ ). However there were significant correlations analysis results between the two different types of

skeletal muscle. Although in the Control group there was no significant correlation in redox status indices between two different types of skeletal muscle ( $p>0.05$ ), in the Uremic group there was significant correlation between psoas and soleus muscle in PC levels ( $r=0.913$ ,  $p=0.002$ ), in GSSG levels ( $r=0.766$ ,  $p=0.027$ ) levels and in CAT levels ( $r=0.743$ ,  $p=0.035$ ) respectively.

**Conclusion:** In our study we found that blood levels of the redox status indices examined did not reflect psoas or soleus muscle concentrations. However, we observed that chronic renal insufficiency disturbed skeletal muscle's redox status in such a way as to create associations between psoas and soleus, two muscles with distinct metabolic profiles, in indices such as PC, GSSG and CAT, which were not evident in control muscles. Certainly, generalised conclusions based only on a single study cannot be drawn, and further studies are needed to verify or not these findings. based on our observations, caution should be taken when interpreting the literature and designing further studies to examine the contribution of oxidative stress in the pathophysiology of chronic disease, especially chronic renal insufficiency.

## Introduction

Oxidative stress has been implicated in several pathological conditions and constitutes a major factor for the progression of many diseases. Thus already, at least in the private sector of health care, companies have developed various diagnostics to trace redox imbalances (e.g. RedoxSYS<sup>®</sup> System, Oxidative Stress Analysis 2.0). Oxidative (and nitrosative) stress also holds great interests for exercise biochemists and physiologists as exercise training adaptations in healthy individuals are reflected by improvements in antioxidant capacity (Nikolaidis et al., 2007; Powers, Sollanek, Wiggs, Demirel, & Smuder, 2014; Soares et al., 2015). Moreover, the likelihood to develop overtraining (Tanskanen, Atalay, & Uusitalo, 2010) appears to be mediated by redox fluctuations, something that holds great interest for coaches and companies providing scientific support to athletes. In recent years, a variety of laboratory tests has been developed for the assessment of redox status indices in biological systems (Jackson, 1999) (de Zwart, Meerman, Commandeur, & Vermeulen, 1999; Holley & Cheeseman, 1993) but also a wide range of ‘applied’ testing services has been developed.

In CKD, muscular weakness, muscle wasting and fatigue, collectively termed as uremic myopathy, constitute functional and physiological abnormalities which characterize renal patients (Campistol, 2002). Previously, we (Research paper 2) and others (reviewed in (Kaltsatou et al., 2015)), have highlighted a role for oxidative stress in uremic myopathy in few but conclusive examinations of muscle tissue of CKD patients or animals. However, as in exercise studies (Bloomer, Fry, Falvo, & Moore, 2007; Bloomer, Goldfarb, & McKenzie, 2006; Deminice et al., 2010; Paschalis et al., 2007) and clinical literature (Gallou et al., 1994; Rockenbach et al., 2011; Santulli et al., 2013; Sveen et al., 2015) the large majority of studies examining the role of ROS in CKD and discussing comorbidities, such as uremic myopathy, have reported increased levels of oxidative stress by only assessing indices in blood samples of CKD patients (Bober et al., 2010; Dolegowska et al., 2007; Filiopoulos et al., 2009; Guo et al., 2013; Papavasiliou et al., 2005; Sahni et al., 2012; Samouilidou & Grapsa, 2003). Clearly popular blood indices such as TBARS and Protein Carbonyls reflect oxidative damage however there is no information about the

specific tissue from which oxidative stress originates from and if uremic myopathy is implicated.

There can be of course variable origins for the elevated concentrations of oxidative stress indices in blood. As with levels of other metabolic products and byproducts (e.g. glucose, lactate, protein etc), blood levels of ROS, whether at health or disease, at a given time point of sampling, reflect the dynamic balance between ROS generation and ROS removal. Firstly, a chronic systemic condition could result in a generalized elevation of these indices in all tissues of an individual. Secondly, increased levels of oxidative stress indices may reflect ROS production linked to damage in a particular tissue and probably constitute an early sign of disease emergence (e.g. one kidney overworking as the other fails (Shah, Baliga, Rajapurkar, & Fonseca, 2007). Thirdly, increased indices in blood could be due to oxidative damage of the circulatory system per se (Arguelles, Garcia, Maldonado, Machado, & Ayala, 2004a). Still, many studies draw conclusions and form hypothesis with regards to muscle or other organ ROS levels by measuring only blood levels.

This is an issue of importance not only for diagnosis but also for assessing benefit of intervention. For example, antioxidant supplementation, especially in HD patients is aid promising to counteract atrophy (R. Ramos & Martinez-Castelao, 2008; Tarng et al., 2004; Trimarchi et al., 2003). Since that skeletal muscle tissue sampling is difficult to obtain, there is no direct clinical evidence that antioxidant supplementation, could reliably reflect improvements in skeletal muscle redox status rather than a systemic benefit.

In order to clarify whether the evaluation of redox status in less-invasive samples, like blood, reflects the redox status of skeletal muscle, the aim of the present study was to correlate and contrast indices of the glutathione system, as well as markers of lipid peroxidation, protein carbonylation and total antioxidant capacity in the blood and skeletal muscle of rabbits used as an animal model of CKD. Given that muscle's mitochondria generate ROS in large quantities, and that slow muscle contain more mitochondria, we contrasted levels in two muscle types, a fast (psoas) and a slow twitch (soleus), at the two ends of the endurance-power continuum.



## Methods

### Animals and Experimental design

New Zealand white female rabbits (young adult, N=15) with body weight of approximately 3200 kg, were first acclimatized to the laboratory animal unit of Medical School (University of Thessaly, Greece) for 48 hours. The animals were housed in a controlled environment with stable conditions of room temperature (RT) (22–24°C) and lighting (12:12 h light-dark cycle). All rabbits were fed with a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum. All animal procedures, including surgery and euthanasia for this project were approved by the ethics committee of the University of Thessaly (decision 2-2/10-10-2012) and the scientific committee of the University Hospital of Larisa, Greece (decision 1/4-1-2012) and animals were under veterinary care, in accordance to the national directives for the care and the use of laboratory animals.

After acclimatization, surgical procedures were performed (sham operation for control animals - Control group and partial nephrectomy for experimental animals – Uremic group. Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87 % and 13 % respectively (proportion 6,69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg body weight of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anesthesia was achieved by a dose of propofol (10mg/kg BW). Three hours before the intervention, each animal had only access to water and not to food and its weight was measured on a precision scale. Animal temperature was maintained via a heating pad.

The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982 (Gotloib et al., 1982). For the Uremic group nine animals (N=9), underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy ( $\frac{3}{4}$ ) of the right kidney. For the Control group six age-matched animals (N=6) underwent sham operation. Twelve

weeks after surgery, the animals were weighed and then sacrificed by injection of sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, sample collections were done in a blind fashion.

## **Sample Preparation**

### ***Blood sampling and treatment***

Blood samples (5ml) were collected from the rabbits' heart and aorta by a heparinized syringe and were placed into ethylene diamine tetra-acetic acid (K<sub>2</sub>EDTA)-containing tubes (Vacutainer Plus Plastic K<sub>2</sub>EDTA; Becton Dickinson). For plasma collection, blood samples were centrifuged immediately at 1370 × g for 10 min at 4° C and the supernatant was carefully collected, aliquoted in multiple eppendorf tubes, stored at -80°C and thawed only once before analysis. The remained packed erythrocytes were lysed with 1:1 (v:v), distilled water, inverted vigorously, and centrifuged at 4000 × g for 15 min at 4° C. The supernatant, red blood cells (RBCs) lysate designated, collected, aliquoted, stored at -80° C and thawed only once before analysis. Finally, in order to obtain serum, another portion of blood sample (5 ml) was collected and placed into separate tubes containing clot activator, left for 20 min to clot at RT and centrifuged at 1,370 × g, at 4°C for 10 min. The supernatant was collected, aliquoted in eppendorf tubes, stored at -80°C and thawed only once before analysis.

### ***Skeletal muscle sampling***

Psoas and soleus muscle samples were harvested from Control (sham-operated) and Uremic group, were frozen immediately in liquid nitrogen, stored at -80° C and part-thawed once (for homogenization) before final analysis.

### ***Skeletal muscle homogenization***

Tissues samples were thawed excised and kept chilled throughout homogenization. A weight portion of each muscle was washed several times with ice-cold normal saline and was placed into pre-chilled eppendorfs containing cold homogenization buffer (138 mM NaCl, 2,7 mM KCl, 1 mM EDTA, pH 7.4) and a mix of protease inhibitors (1µM aprotinin, 1µg/ml leupeptin and 1mM PMSF). Initial homogenization was achieved with an electrical homogenizer (MICCRA D-9) for 10

minutes with intermediate pauses of 10sec/20 sec. Then an ultrasound homogenizer (UP50H) was used for 1-2 minutes, with intermediate pauses as before. Homogenates were filtered through four layers of medical gauze to remove connective tissue debris, incubated for 10 min at 4° C, and centrifuged at 10,000 X g for 10min again at 4° C. The supernatant was aliquoted in multiple portions and stored at -80° C.

### **Biochemical & Redox Status Indices Analyses**

Biochemical analysis in blood (urea, creatinine) and redox status indices (GSH, GSSG, Ratio GSH/GSSG, GR-Reductase, CAT, TAC, TBARS and PC) analyses in blood as well as in skeletal muscle of control and uremic groups, were analyzed according to the assays described in the previous studies (Research Papers 1&2).

### **Statistical Analysis**

Possible relationships between indices were examined using the Pearson Correlation Coefficient analysis in pool and per group data. The minimum significance level was set at  $P < 0.05$ .

## **Results**

For each marker, the correlation between blood and skeletal muscle was evaluated in order to examine, firstly whether changes in blood levels reflected changes in skeletal muscle levels (whether in the sham-operated control or the uremic condition). Secondly, it was tested if there was evidence of muscle type specificity on the blood levels of the examined redox balance indices.

A very strong and significant correlation between blood urea and creatinine levels, the two critical blood biochemistry markers for the diagnosis of renal insufficiency, in the Uremic group and pool data, but not in the Control group, verified that our animal model successfully mimicked chronic renal insufficiency.

**Table 4.4.1.** Pearson correlation (r) between blood levels of creatinine and urea levels in the pool and group data.

	<u>Pool Data</u> Blood Creatinine	<u>Control</u> <u>Group</u> Blood Creatinine	<u>Uremic</u> <u>Group</u> Blood Creatinine
<u>Pool Data</u> Blood UREA	r=0.920* p=0.000		
<u>Control Group</u> Blood UREA		r=0.779 p=0.431	
<u>Uremic Group</u> Blood UREA			r=0.926* p=0.001

For each one of the antioxidant or oxidative stress indices, statistical analysis didn't show any significant correlation between blood and the two examined muscles' levels neither in the pool of samples nor for each group separately ( $p > 0.05$ ).

**Table 4.4.2.** Pearson correlation (r) between blood and skeletal muscle levels of redox indices in pool data, and separately in the Control and Uremic groups, psoas and soleus. Exact significance levels (p) are reported.

	GSH	GSSG	GSH/G SSG	GR- Reductase	CAT	TAC	TBARS	PC
<u>Pool Data</u> Blood & Psoas	r=0.123 p=0.719	r=0.194 p=0.567	r=0.077 p=0.822	r=0.100 p=0.757	r=0.435 p=0.137	r=0.066 p=0.832	r=0.332 p=0.268	r=0.034 p=0.913
<u>Pool Data</u> Blood & Soleus	r=0.059 p=0.862	r=0.518 p=0.103	r=0.532 p=0.092	r=0.553 p=0.077	r=0.093 p=0.761	r=0.400 p=0.176	r=0.402 p=0.174	r=0.008 p=0.978
<u>Control Group</u> Blood & Psoas	r=0.359 p=0.641	r=0.408 p=0.592	r=0.071 p=0.929	r=0.451 p=0.446	r=0.311 p=0.611	r=0.416 p=0.486	r=0.214 p=0.730	r=0.480 p=0.413
<u>Control Group</u> Blood & Soleus	r=0.229 p=0.771	r=0.543 p=0.457	r=0.221 p=0.779	r=0.639 p=0.361	r=0.449 p=0.448	r=0.039 p=0.951	r=0.726 p=0.165	r=0.740 p=0.153
<u>Uremic Group</u> Blood & Psoas	r=0.004 p=0.993	r=0.089 p=0.850	r=0.197 p=0.671	r=0.152 p=0.745	r=0.152 p=0.745	r=0.503 p=0.204	r=0.776 p=0.203	r=0.385 p=0.347
<u>Uremic Group</u> Blood & Soleus	r=0.388 p=0.389	r=0.452 p=0.309	r=0.727 p=0.164	r=0.538 p=0.213	r=0.538 p=0.213	r=0.519 p=0.187	r=0.452 p=0.261	r=0.174 p=0.680

GSH: reduced glutathione; GSSG: oxidized glutathione; TAC: total antioxidant activity; TBARS: thiobarbituric acid-reactive substances; PC: protein carbonyls,

Moreover, we examined the correlation between the critical blood biochemistry markers for the diagnosis of renal insufficiency, creatinine and urea, pool and group levels, with blood levels of the redox indices examined. While no relationship reached statistical significance it is noteworthy that in the pool data Creatinine levels tended to correlate with blood GSH ( $r=0.664$ ,  $p=0.073$ ). Likewise, Urea tended to correlate with blood GSH ( $r=0.655$ ,  $p=0.078$ ).

**Table 4.4.3.** Pearson correlation (r) between blood urea and creatine and blood levels of the redox indices examined in pool data, Control and Uremic group.

	GSH	GSSG	GSH/G SSG	GR- Reductase	CAT	TAC	TBARS	PC
<u>Pool Data</u> Urea	r=0.655 p=0.078	r=0.019 p=0.965	r=0.438 p=0.277	r=0.132 p=0.716	r=0.101 p=0.769	r=0.038 p=0.911	r=0.181 p=0.595	r=0.125 p=0.713
<u>Pool Data</u> Creatinine	r=0.664 p=0.073	r=0.073 p=0.864	r=0.339 p=0.411	r=0.197 p=0.586	r=0.031 p=0.929	r=0.143 p=0.676	r=0.067 p=0.844	r=0.005 p=0.988
<u>Control Group</u> Urea	r=0.113 p=0.928	r=0.241 p=0.845	r=0.062 p=0.961	r=0.988 p=0.099	r=0.293 p=0.811	r=0.375 p=0.755	r=0.419 p=0.724	r=0.942 p=0.218
<u>Control Group</u> Creatinine	r=0.534 p=0.641	r=0.420 p=0.724	r=0.673 p=0.530	r=0.673 p=0.530	r=0.371 p=0.758	r=0.754 p=0.456	r=0.896 p=0.293	r=0.523 p=0.649
<u>Uremic Group</u> Urea	r=0.614 p=0.195	r=0.180 p=0.733	r=0.641 p=0.170	r=-0.204 p=0.661	r=0.062 p=0.883	r=0.204 p=0.628	r=0.312 p=0.452	r=0.160 p=0.705
<u>Uremic Group</u> Creatinine	r=0.495 p=0.319	r=0.262 p=0.616	r=0.683 p=0.135	r=-0.210 p=0.651	r=0.346 p=0.401	r=0.417 p=0.305	r=0.252 p=0.547	r=0.034 p=0.936

GSH: reduced glutathione; GSSG: oxidized glutathione; TAC: total antioxidant activity; TBARS: thiobarbituric acid-reactive substances; PC: protein carbonyls,

Furthermore, we examined the correlation between the blood biochemistry markers, creatinine and urea, pool and group levels, with muscle levels of the redox indices examined. Neither in the Control nor in the Uremic group were there any statistically significant correlations observed between these two markers and soleus or psoas muscle levels of the redox indices.

**Table 4.4.4.** Pearson correlation (r) between blood urea and creatinine and psoas skeletal muscle redox status indices in pool data, Control and Uremic group.

PSOAS	GSH	GSSG	GSH/G SSG	GR-Reductase	CAT	TAC	TBARS	PC
<u>Pool Data</u> Urea	r=0.381 p=0.277	r=-0.372 p=0.291	r=0.205 p=0.569	r=0.398 p=0.254	r=0.104 p=0.775	r=0.264 p=0.462	r=0.111 p=0.759	r=0.222 p=0.537
<u>Pool Data</u> Creatinine	r=0.353 p=0.317	r=0.420 p=0.227	r=0.338 p=0.340	r=0.393 p=0.262	r=0.034 p=0.926	r=0.269 p=0.452	r=0.49 p=0.892	r=0.349 p=0.322
<u>Control Group</u> Urea	r=0.113 p=0.918	r=0.241 p=0.845	r=0.162 p=0.961	r=0.566 p=0.617	r=0.890 p=0.305	r=0.385 p=0.715	r=0.951 p=0.201	r=0.935 p=0.218
<u>Control Group</u> Creatinine	r=0.534 p=0.641	r=0.420 p=0.724	r=0.573 p=0.130	r=0.075 p=0.952	r=0.408 p=0.733	r=0.873 p=0.324	r=0.547 p=0.632	r=0.323 p=0.609
<u>Uremic Group</u> Urea	r=0.592 p=0.162	r=0.516 p=0.236	r=0.108 p=0.818	r=-0.622 p=0.136	r=0.280 p=0.544	r=0.362 p=0.424	r=0.484 p=0.271	r=0.047 p=0.920
<u>Uremic Group</u> Creatinine	r=0.579 p=0.173	r=0.607 p=0.149	r=0.230 p=0.620	r=-0.679 p=0.194	r=0.307 p=0.503	r=0.393 p=0.383	r=0.493 p=0.261	r=0.101 p=0.829

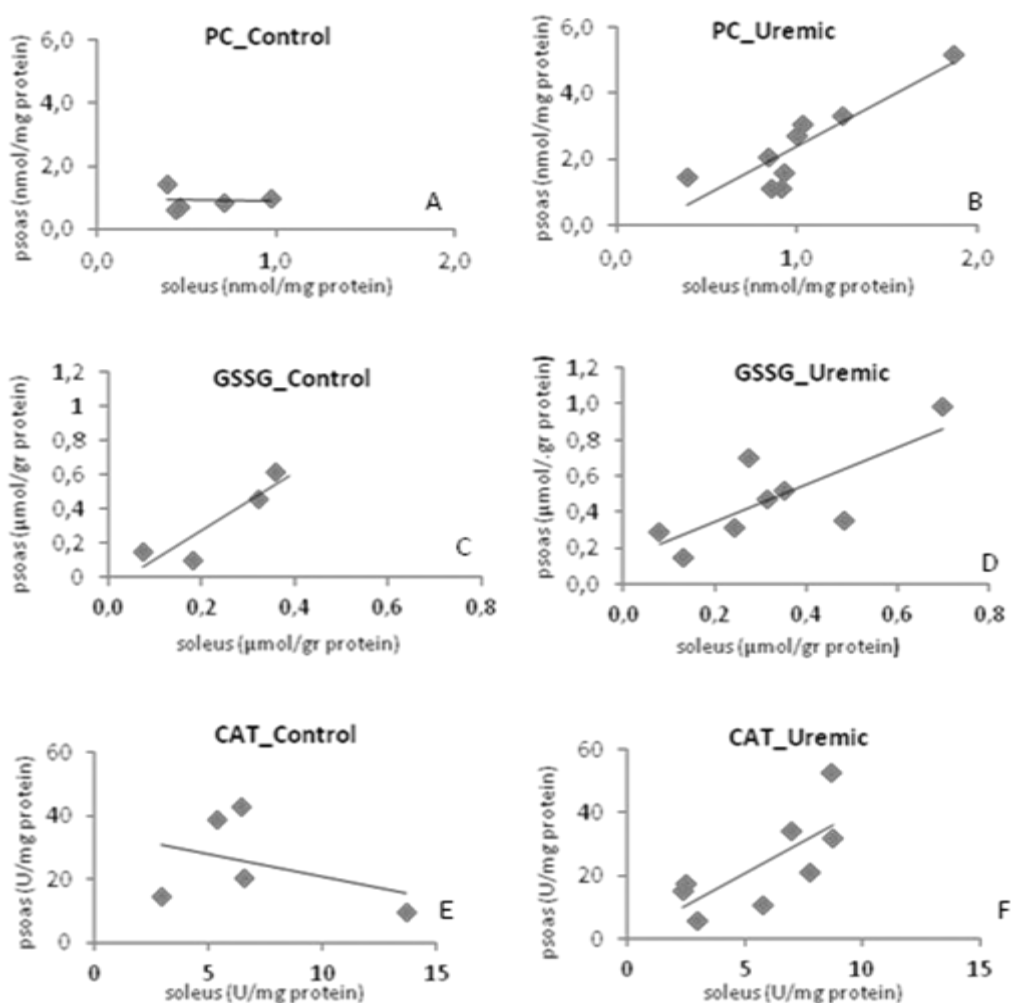
GSH: reduced glutathione; GSSG: oxidized glutathione; TAC: total antioxidant activity; TBARS: thiobarbituric acid-reactive substances; PC: protein carbonyls,

**Table 4.4.5.** Pearson correlation between blood urea and creatinine and soleus skeletal muscle redox status indices in pool data, Control and Uremic group

SOLEUS	GSH	GSSG	GSH/G SSG	GR-Reductase	CAT	TAC	TBARS	PC
<u>Pool Data</u> Urea	r=0.345 p=0.364	r=0.145 p=0.709	r=0.083 p=0.277	r=0.216 p=0.577	r=0.437 p=0.207	r=0.019 p=0.959	r=0.215 p=0.551	r=0.033 p=0.928
<u>Pool Data</u> Creatinine	r=0.419 p=0.262	r=0.172 p=0.658	r=0.150 p=0.700	r=0.148 p=0.703	r=0.367 p=0.297	r=0.232 p=0.520	r=0.250 p=0.485	r=0.072 p=0.842
<u>Control Group</u> Urea	r=0.055 p=0.945	r=0.162 p=0.838	r=0.439 p=0.561	r=0.673 p=0.530	r=0.972 p=0.152	r=0.725 p=0.483	r=0.962 p=0.177	r=0.674 p=0.529
<u>Control Group</u> Creatinine	r=0.900 p=0.100	r=0.213 p=0.787	r=0.233 p=0.767	r=0.173 p=0.742	r=0.906 p=0.279	r=0.134 p=0.914	r=0.921 p=0.254	r=0.988 p=0.0198
<u>Uremic Group</u> Urea	r=0.380 p=0.401	r=0.246 p=0.594	r=0.181 p=0.698	r=-0.300 p=0.514	r=0.612 p=0.144	r=0.300 p=0.513	r=0.170 p=0.716	r=0.258 p=0.577
<u>Uremic Group</u> Creatinine	r=0.457 p=0.302	r=0.341 p=0.455	r=0.323 p=0.479	r=-0.274 p=0.552	r=0.590 p=0.163	r=0.281 p=0.541	r=0.221 p=0.633	r=0.318 p=0.487

GSH: reduced glutathione; GSSG: oxidized glutathione; TAC: total antioxidant activity; TBARS: thiobarbituric acid-reactive substances; PC: protein carbonyls,

However there were significant correlations analysis results between the two different types of skeletal muscle and in a way indicative of disease-induced modifications. Although in the Control group there was no significant correlation in redox status indices between two different types of skeletal muscle ( $p>0.05$ ), in the Uremic group there was significant correlation between psoas and soleus muscle in PC levels ( $r=0.913$ ,  $p=0.002$ ), in GSSG levels ( $r=0.766$ ,  $p=0.027$ ) levels and in CAT levels ( $r=0.743$ ,  $p=0.035$ ) respectively (Fig. 4.4.1).



**Fig.4.4.1.** Correlation of redox indices between the two different types of skeletal muscle studied for CON (left) and UREM (right) samples. PC concentration, A: no significant correlation B:  $r=0.913$ ,  $p=0.002$ . GSSG concentration C: no significant correlation, D:  $r=0.766$ ,  $p=0.027$ . CAT activity, E: no significant correlation, F:  $r=0.743$ ,  $p=0.035$  between psoas and soleus muscle in Control and Uremic group respectively.

## Discussion

In the present study we observed that there was no correlation between the redox indices evaluated in the blood and their levels in the two different types of skeletal muscle (psoas, soleus). However we observed significant correlations in critical redox indices between the two different types of skeletal muscle only in the Uremic group indicating a disease-induced modification, in agreement to previous results. Also, a tendency existed for key biochemical indices, such as urea and creatinine, to correlate with blood redox status markers in the Uremic samples but not in the Control.

As it is the case with levels of other metabolic products and byproducts (e.g. glucose, lactate, protein etc), blood levels of ROS at a given time point of sampling, whether in health or disease, reflect the *dynamic balance* between ROS generation and ROS neutralization. The origin of the ROS (or RNS), and their levels at the point of origin cannot be easily surmised. If a disease state is implicated then not only the primarily suffering organic system could be contributing to a redox imbalance (as e.g. in kidney disease (Shah et al., 2007) but also secondarily affected systems (e.g. muscle), as well as systemic inflammation (Lim, Cheng, et al., 2002; Silverstein, 2009) and vascular stress (Arguelles, Garcia, Maldonado, Machado, & Ayala, 2004b) could be implicated.

In this study, by employing an animal model of CKD, we sought to examine whether the increased levels of oxidative stress observed in the blood (Research Paper 1) reflect perhaps the muscle levels of redox indices (Research Paper 2).

We found no correlations between blood and skeletal muscle levels of redox indices either in the pool data, or separately in the Control and Uremic groups, for psoas and soleus muscle samples. In a previous study (Research Paper 2) we observed that PC levels were significantly increased in both muscle types of the UREM samples and that soleus muscle (whether in Control or Uremic) presented with significantly higher GSH, CAT and Gr-reductase but also TBAR levels compared to psoas. Our blood analysis did not reflect the muscle PC levels nor any muscle type specific attributes. It is possible that the development of uremic myopathy (and thus skeletal muscle's contribution to oxidative stress) was not so advanced as to be



reflected in blood levels. A study from our group (Mitrou, Galler, & Karatzaferi, 2015) indicated a moderate atrophy of about 11% in psoas muscle and we have no data on soleus. Based on human studies however we expect that fast muscles are the ones mostly affected by atrophy (Sakkas, Ball, et al., 2003) and the increased protein carbonylation observed in our psoas muscle re-iterate the human observations.

The lack of correlations between redox indices' levels in blood and muscle samples in our study do not agree with a recent rat study. Veskoukis et al (2009) reported that four redox status indices (PC, GSH, GSSG, Catalase) in blood could adequately reflect the redox status at rest, after exercise and/or following xanthine oxidase inhibition in skeletal muscle (gastrocnemius, which has a different myosin composition than rabbit psoas) (Veskoukis, Nikolaidis, Kyparos, & Kouretas, 2009). Moreover, blood GSSG was found in that study to correlate strongly with muscle, liver and heart GSSG levels. Animal model, techniques differences and the renal dysfunction it is possible that may have an overarching systemic effect that could mask muscle's contribution to blood levels of redox indices in our data. Likewise, it is possible that the sham-operation also played a similar role. Moreover we should also note that it is not clear if in the study by Veskoukis et al the analysis for GSSG included an alkylating agent to preserve its levels, as done in the present work (Research Paper 1&2) thus precluding comparison due to methodological differences.

As already discussed (Research Paper 3) in the literature for the majority of patient studies redox status indices are evaluated in blood and results are extrapolated to tissues. Rodriguez et al (2012) found that protein carbonyls concentrations moderately correlated between blood and skeletal muscle (vastus lateralis, a mixed muscle) in patients with chronic obstructive pulmonary disease (Rodriguez et al., 2012). We cannot exclude that in advanced CKD patients PC muscle levels may be also reflected in blood. The increased protein carbonylation observed in our psoas muscle re-iterate the human observations and perhaps indicate that increased muscle PC levels is one of the earliest redox modifications occurring in uremic muscle.

The present study was the first to evaluate the relationships between redox status indices in the blood and skeletal muscle under renal insufficiency. Notably, we observed that none of the redox status indices examined in the two different types of skeletal muscle, was reliably reflected in the blood. Based in the above findings, it seems that there is no correspondence between blood and skeletal muscle redox status indices in CKD, demonstrating that tissue specific redox status changes do not cause

corresponding changes that can be measured in blood, unfortunately inhibiting our ability to use blood testing to monitor muscle redox status in pre-dialysis patients.

However, we also examined if disease severity, as reflected in blood levels of creatinine and urea are associated with either blood or muscle redox status. We observed a tendency for Creatinine and Urea blood levels in the pool data to correlate with blood GSH. We have previously discussed that blood GSH levels are upregulated as an adaptive response to disease (Research Paper 1), in agreement to human studies (Bober et al., 2010). Perhaps, if verified by further work, clinicians which use blood creatinine and urea levels to monitor CKD progression could also develop threshold values to flag up redox disturbances.

Significant correlations of redox status indices among the two types of skeletal muscle (psoas, soleus), were found only for the Uremic group and not for the Control group, in PC, GSSG and CAT levels between psoas and soleus muscle levels. This discrepancy indicated that the experimentally induced chronic renal insufficiency posed an appreciable redox challenge for skeletal muscle in the Uremic groups, whose samples presented with a coordinated variability in redox levels (in contrast to Control). Specifically, increasing GSSG and PC levels (the latter significantly increased in both Uremic soleus and psoas vs Controls – Research Paper 2) indicated disease-induced oxidative damage in both muscle types, which in turn stimulated the activation of the antioxidant enzyme CAT (found to be significantly higher in soleus muscles overall – Research Paper 2).

## Conclusion

In our study we found that blood levels of the redox status indices examined (PC, GSH, GSSG, CAT, Cr-Reductase, TBARS, TAC) did not reflect psoas or soleus muscle concentrations. However, we observed that chronic renal insufficiency disturbed skeletal muscle's redox status in such a way as to create associations between psoas and soleus, two muscles with distinct metabolic profiles, in indices such as PC, GSSG and CAT, which were not evident in control muscles. Moreover, critical biochemistry indices such as creatinine and urea tend to associate with blood GSH. Certainly, generalised conclusions based only on a single study cannot be drawn, and further studies are needed to verify or not these findings. Moreover, it is possible that other muscle redox indices, not examined here (such as advanced oxidation protein products AOPSS, F2 isoprostane, advanced glycosylation end-product AGE (Drueke et al., 2002)), could have a better reflection in blood levels in pre-dialysis CKD. However, we examined redox markers which are popular and affordable. Thus, based on our observations, caution should be taken when interpreting the literature and designing further studies to examine the contribution of oxidative stress in the pathophysiology of chronic disease, especially chronic renal insufficiency.

## **5. General Discussion**

This PhD Thesis is a modest contribution to the ongoing discussion about “Redox status in Chronic Kidney disease” and its effects on patients. We used an animal model of renal disease (surgically induced partial nephrectomy in New Zealand rabbits) where confounding factors, common in human studies, such as comorbidities and pharmacological treatment are not implicated. Renal insufficiency was induced by removal of the right kidney and partial nephrectomy of the left kidney (for the Uremic group and sham-operation for Control animals).

Specifically, we evaluated a variety of redox status indices, oxidative stress indices as well as antioxidant molecules and enzymes in blood and in two different types of skeletal muscle, in the fast glycolytic psoas and in the slow oxidative soleus. Moreover, we evaluated the effects of CKD on biochemical and hematological status. We also examined whether the oxidative stress markers measured in blood adequately reflects redox status in skeletal muscle and whether redox status in psoas muscle adequately depicts the redox status in soleus muscle. Moreover, it was examined if blood urea and creatinine levels had any possible reflection on blood and skeletal muscle redox status and if there is a correlation between urea and creatinine levels.

Regarding redox status analysis in blood, our major findings indicated that GSH concentration was significantly higher in the erythrocytes of the Uremic group compared to the Control and that there was a tendency for an increase in TBARS concentration in the plasma of Uremic group compared to Control. The rest of the redox status indices did not show any significant differences or tendencies between the Control and the Uremic group.

As far as the redox status evaluation in skeletal muscle is concerned, our study addressed increased PC levels in skeletal muscle, by approximately 1.82 fold for psoas and 2.71 fold for soleus in Uremic compared to Control samples. Moreover, we observed significant differences between the two skeletal muscle types (psoas and soleus) studied. Specifically, Total protein and TAC concentrations were found to be decreased in soleus muscle compared to psoas in both Control and Uremic group. Additionally, soleus demonstrated higher levels of TBARS and PC levels as well as higher GSH levels, Catalase and GR-Reductase activities compared to psoas muscle in both the Control and Uremic group with Uremic soleus to demonstrate further increased GSH levels and GR-Reductase and CAT activities.

Additionally our study systematically examined a range of biochemical and hematological indices. Results indicated that there was no change in blood lipid and glucose profile of the Uremic group as compared to the Control group.

However, there were significant differences in hematological parameters between the Control and the Uremic group. RBC, hematocrit and MCH levels were found to be decreased in the Uremic group and MCV had a tendency to decrease in the Uremic group compared to the Control group. Overall, in the Uremic group, the RBC, Hemoglobin, hematocrit and MCH levels were at the lower limits of physiological reference values.

Finally, in the present study we observed that there was no correlation between the redox indices evaluated in the blood and their levels in the two different types of skeletal muscle (psoas, soleus) respectively. However we observed significant correlations in critical redox indices (PC, GSSG and CAT) between the two different types of skeletal muscle only in the Uremic group. Also, a tendency existed for key biochemical indices, such as urea and creatinine, to correlate with the blood antioxidant molecule (GSH) in the Uremic samples but not in the Controls.

As far as redox status evaluation in blood is concerned, past studies suggested that oxidative stress is implicated in CKD pathophysiology. There is evidence that as the kidney function is getting worse, the redox status imbalance becomes more profound. However, very limited information existed about redox changes in early CKD stages and whether antioxidant capacity or ROS overproduction (or both) may be altered. Our study tries to answer such open questions.

There is an ambivalence with regards to GSH blood levels in human patient studies (having been found lower (Drai et al., 2001; Lim, Cheng, et al., 2002; Schettler et al., 1998) or higher (Biasioli et al., 1996; Bober et al., 2010; Stepniowska et al., 2006) in various HD studies). In moderately uremic pre-dialysis patients, in agreement to our observations, Bober et al showed higher levels of GSH in whole blood of CKD patients compared to age-matched healthy (Bober et al., 2010) (which increased further in HD patients). However, in other studies a reduction in GSH levels in the whole blood of CKD patients has been observed compared to controls (Annuk et al., 2001; Ceballos-Picot et al., 1996; Sahni et al., 2012) reflecting a depletion in the antioxidant reserve. Overall, the increased GSH blood levels in our study could be the result of an adaptive response to the uremic environment. The latter appears more likely in our case given the almost doubling of blood GSSG in the Uremic group

(Uremic levels being 1.77 fold of those of Controls) which, without reaching statistical significance, indicated increased levels of hydrogen peroxide or lipid peroxides, similarly to human studies (Annuk et al., 2001).

Nonetheless, catalase also reduces hydrogen peroxide to water but there was no statistical difference in the activity of the specific antioxidant enzyme between the Control and the Uremic group (rather its activity tended to be lower in erythrocytes of the Uremic group). Based on the above, it appeared that hydrogen peroxide scavenging was undertaken in a greater degree by the glutathione redox cycling mechanism than catalase in our CKD model.

The significant increase of GSH in uremic blood samples could also explain the observed, largely undisturbed, TAC and carbonylation levels of blood proteins. In our study animals followed the same diet, carefully designed not to tax the remaining kidney function, similarly to diet guidelines followed by patients, while providing balanced nutrients and minerals. However, dietary intakes can greatly influence GSH levels, and could explain the literature's conflicting reports with regards to human patients' GSH levels.

In our work we developed our methodology (data not shown) to prevent artifacts during GSH and GSSG titration, with the most important one being a 5-15% oxidation of GSH during sample deproteination with acids. This artifact can lead to remarkable overestimation of GSSG (Rossi et al., 2002) and underestimation of total GSH. Thus we applied the thiol alkylating agent NEM at the preparatory stages to prevent the oxidation of GSH. In the conflicting patient studies on GSH and GSSH findings (Annuk et al., 2001; Bober et al., 2010; Ceballos-Picot et al., 1996; Sahni et al., 2012) it is not clear if such measures were always taken. Notwithstanding the above, it should be also noted that blood levels of GSH, as a 'systemic' marker, cannot reveal its origins. However, its increased levels in circulation confer an antioxidant advantage preventing or ameliorating vascular and other damage.

The increased (but not statistically significant) plasma TBARS levels by approximately 1.4 fold, in Uremic compared to Control samples, point to increased levels of lipid peroxidation, a process where oxidants, such as free radicals, assault lipids and especially their membranes (Ayala et al., 2014), leading to alterations of cell's quality and survival. Regarding HD patients, the large majority of studies reported increased TBARS levels in plasma compared to healthy individuals (Bober et al., 2010; Dimitrijevic et al., 2012; Guo et al., 2013; Haklar et al., 1995; Sakata et

al., 2008; Sommerburg et al., 1998), reflecting extensive lipid peroxidation. Taking all the above into consideration together with our findings, it could be concluded that lipid peroxidation in CKD emerges even from the early stages and is getting worse during the progression of the disease.

Given that skeletal muscle contains high levels of fibrillar proteins, and that their turnover is quite slow, skeletal muscle becomes extremely susceptible to free radical oxidation with adverse functional outcomes. We observed increased protein carbonylation in both muscle types of our Uremic group. Our findings are in agreement with those reported by Lim et al (Lim, Cheng, et al., 2002) in skeletal muscle of uremic patients on HD. The increased levels of PC in a pre-dialysis stage of CKD, as it was observed in our study, could be regarded as a very interesting finding for the time point of protein oxidation, revealing the early emergence of oxidative damage to protein content. Taking into account the role of carbonyl stress in vascular damage (Chen et al., 2013) and the general functionality of muscle, an early countermeasure for protein oxidation could be of great importance for patients before they move into the end-stage where the negative effects on muscle status may become irreversible.

Skeletal muscle is a heterogeneous tissue which consists of a large diversity of fiber types. This heterogeneity in muscle type composition determines distinct biochemical and structural characteristics which in turn are responsible for the variety of physiological and metabolic functions (Essen-Gustavsson et al., 1992; Pette & Staron, 1990). In our study we examined the redox status of two different types of skeletal muscle, the slow twitch soleus and the fast twitch psoas in order to observe their possible different response in the uremic environment. Consistently to the two muscle type differences in mitochondrial content, TBARS levels were higher in soleus muscles than psoas, irrespective of group.

Among other pathways, muscle catabolism is mediated by TNF- $\alpha$ /NF- $\kappa$ B signaling which in turn is affected from endogenous ROS. TNF- $\alpha$  widely excites mitochondria ROS production, promoting TNF- $\alpha$ /NF- $\kappa$ B activation (Hennet et al., 1993; Schulze-Osthoff et al., 1992), a process which seems to be tissue specific (Y. P. Li et al., 1998). Taking into consideration that soleus muscle contains a higher amount of mitochondria, it would be rather logical that it would be more sensitive to NF- $\kappa$ B activation and therefore, to protein loss and muscle wasting. Our findings, where soleus muscle exhibited lower total protein levels, seem to confirm this observation.



Based on the increased amount of mitochondria in slow muscles (Engel, 1970), it could be expected that soleus may be more exposed to ROS and their negative effects, but also that it may develop an appropriate antioxidant capacity to withstand the oxidative load. Despite the reduced TAC levels in soleus muscle in comparison with psoas, the increased levels GSH, CAT and CR-reductase indicate that healthy soleus indeed demonstrates a higher antioxidant capacity than psoas, with uremic soleus appearing to have likewise further upregulated its defenses.

We observed no changes in lipid profile between the Control and the Uremic group to point to the development of dyslipidemia (as usually observed in human patients). Perhaps more time was needed for our model of uremia to develop such changes in Cholesterol. Furthermore, diet composition is known to influence blood biochemical parameters and the animals of the present study were fed the same diet (3.5% fat content). Thus differences in our methods and species could also contribute to our results. Still, the observed tendency for increased lipid peroxidation (TBARS) in the plasma of the Uremic compared to the Control and the tendency for a higher Triglycerides/HDL ratio in the Uremic group fits well with a progressive atherogenic development due to chronic renal insufficiency.

However, there were significant differences in the levels of hematological parameters in the Uremic group as compared to the Control group. RBC count and Hematocrit levels were found to be decreased in the Uremic group. The mean corpuscular hemoglobin or mean cell hemoglobin (MCH) was significantly lower in the Uremic group. MCH reflects the average mass of hemoglobin per red blood cell, and lower values may be linked to hypochromic anemias and microcytic RBC. The latter is possible as we observed a tendency for lower mean corpuscular volume or mean cell volume (MCV), in the Uremic group. A possible explanation for the aforementioned hematological disturbances could be the expected impairment in the erythropoietin production and other factors which influence negatively the red cell production in bone marrow and the red cell lifespan. Moreover, apoptosis may be increased and contribute to the disturbed hematological status. A higher apoptotic rate could be hypothesized by the higher bilirubin levels in the experimental group. Overall, taking into consideration the fact that Hb, Hct and MCH levels and red blood cell count in the Uremic group were at the lower limits of rabbit physiological values it is concluded that these findings constitute early signs of anemia development. As previously discussed, despite USA and EU guidelines for the administration of EPO

in pre-dialysis stage patients this approach is not so much popular since anemia development may not be clear in the pre-dialysis patients. Our findings of low hematocrit and overall disturbed hematological parameters in an animal model of pre-dialysis CKD further verify that our model represents an (untreated) human renal disease condition (when EPO is not supplemented). Taken together, our observations and the human data, and guidelines, these findings indicate that anemia could have a major role in disease development and symptoms severity and should be addressed as early as possible.

The present study was the first to evaluate the relationships between redox status indices in the blood and skeletal muscle under renal insufficiency. It is known that blood levels of ROS at a given time point of sampling, whether in health or disease, reflect the *dynamic balance* between ROS generation and ROS neutralization. The origin of the ROS (or RNS), and their levels at the point of origin cannot be easily surmised. If a disease state is implicated then not only the primarily suffering organic system could be contributing to a redox but also secondarily affected systems (e.g. muscle), as well as systemic inflammation and vascular stress could be implicated. Notably, we observed that none of the redox status indices examined in the two different types of skeletal muscle, was reliably reflected in the blood. Based in the above findings, it seems that there is no correspondence between blood and skeletal muscle redox status indices in CKD, demonstrating that tissue specific redox status changes do not cause corresponding changes that can be measured in blood. Moreover, it is possible that the development of uremic myopathy (and thus skeletal muscle's contribution to oxidative stress) was not so advanced as to be strongly reflected in blood levels. We cannot exclude that in advanced CKD, PC muscle levels may be also reflected in blood. The increased protein carbonylation observed in our psoas muscle re-iterate the human observations and perhaps indicate that increased muscle PC levels is one of the earliest redox modifications occurring in uremic muscle that could presumably be reflected in blood at a later stage. Perhaps the upregulation of blood GSH levels observed, acted to neutralize ROS in the blood inhibiting our ability to trace muscle redox status in uremic muscle. This, assumption is strengthened by the observation for a tendency for Creatinine and Urea blood levels in the pool data to correlate with blood GSH. Creatinine and urea reflect kidney disease severity. Perhaps, if verified by further work, clinicians which use blood

creatinine and urea levels to monitor CKD progression could also develop threshold values to flag up redox disturbances.

The two muscle types are expected, due to the known differences in oxidative capacity, to have a different redox profile. Significant correlations of redox status indices among the two types of skeletal muscle (psoas, soleus), were found only for the Uremic group and not for the Control group, in PC, GSSG and CAT levels between psoas and soleus muscle levels. This discrepancy indicated that the experimentally induced chronic renal insufficiency posed an appreciable redox challenge for skeletal muscle in the Uremic groups, whose samples presented with a coordinated variability in redox levels (in contrast to Control).

### Limitations of the study

In the current research some potential weaknesses have been recognized that need to be acknowledged. Firstly, a bigger number of animals could perhaps give a more clear aspect of the tendencies for some indices examined, perhaps also including a non-operated control group. However, due to the high costs of the model and ethical considerations this was not possible.

Secondly the evaluation of additional indices of oxidative damage such as 8-OH-dG for DNA oxidative damage, isoprostanes for lipid peroxidation, advanced glycosylation end-products (AGEs) and advanced oxidation protein products (AOOPs) could perhaps add a more detailed picture of the redox status. We however, focused on popular techniques, reliably used in our and other laboratories.

The lack of insulin sensitivity evaluation, and technical difficulties with regards to blood and muscle glycogen analysis, restricted our ability to address the possibility of the metabolic syndrome development in early CKD. Moreover, as we did not measure oxidized LDL we could not strongly address atherogenicity. Last but not least it was not possible to measure Erythropoietin levels in our animal model.

## **6. Concluding Remarks**

In conclusion, the results of this work demonstrate that even in the pre-dialysis stages of CKD there is an emergence of oxidative stress in blood and as an adaptive response the antioxidant defense mechanisms are upregulated in order to preserve redox status homeostasis. Moreover in both types of skeletal muscle (psoas, soleus) carbonyl formation indicates an early stimulus of muscle protein degradation. However both oxidative damage and antioxidant response seem to be muscle type-specific. Our model of CKD shows that the lipid profile in this stage of the disease development does not seem to be disturbed to a great extent. However, an atherogenic development cannot be excluded and should be addressed early on. Still, we found a severely disturbed hematological profile in experimental animals. This highlights that early recognition of anemia, in pre-dialysis stage, could play a key role in its treatment and could probably delay the progression of CKD. Last but not least, it was found that blood levels of the redox status indices did not reflect muscle concentrations and more work is needed in the direction of less invasive monitoring of muscle related redox imbalances.

Overall, our results highlight the need for interventions early during disease progression in order to protect skeletal muscle quality, maintain redox balance and correct anemia, and thus allow patients to reach the end-stage at the best possible status.

### Future perspectives:

To complement these observations future work should investigate modified LDLs and insulin sensitivity in order to address the possibility of the metabolic syndrome development in early CKD.

Our findings should be verified in human studies. While this is difficult to achieve due to ethical and practical constraints perhaps opportunity sampling (e.g. during fistula installation) could allow for muscle sample analysis in an advanced CKD stage.

Last but not least, the lack of correlation between levels of blood and muscle indices doesn't preclude the possibility that other redox markers or at a different disease state, less invasive blood diagnostics could be developed.

Another issue that probably should be discussed is the presence of two fundamental biological processes, autophagy and inflammation, which are involved in both physiological and pathological conditions. It has been suggested that CKD occurs partly due to imbalance between the molecular mechanisms that govern oxidative stress, inflammation, autophagy and cell death. Mounting evidence suggests that both oxidative stress and autophagy are significantly involved in CKD. However, limited information is available for understanding signaling pathways of autophagy and oxidative stress. As it is known, basal levels of redox signaling and autophagy signaling are necessary to preserve cellular homeostasis and to trigger cytoprotective pathways. Changes in autophagic flux have been shown to regulate ROS production. Similarly, several lines of evidence suggest that free radicals act as upstream modulators of autophagy induction. Therefore, it is believed that there exists a two-way interaction between oxidative stress and autophagy, but there are important questions that still need to be answered. The specific signals regarding the molecular interactions between oxidative stress and autophagy remain to be uncovered. Specific therapeutic strategies that can regulate both oxidative stress and autophagy signaling are needed to benefit patients with CKD.

Inflammation process is regarded as the most common outcome of oxidative stress. ROS directly or indirectly can elevate inflammation, and trigger the expression of pro-inflammatory cytokines. Although the lack of inflammation indices' evaluation

in our study could be considered as a limitation, the levels of leukocytes, a clinical marker of systemic inflammation, were between the normal ranges in both groups



## 7. References

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## **APPENDICES**

## APPENDIX A

### Υπεύθυνη Δήλωση

Η κάτωθι υπογεγραμμένη Κωνσταντίνα Πουλιανίτη με ΑΕΜ 16/2011, διδακτορική φοιτήτρια της Σχολής Επιστήμης Φυσικής Αγωγής και Αθλητισμού του προγράμματος Άσκηση και Υγεία

δηλώνω υπεύθυνα ότι αποδέχομαι τους παρακάτω όρους που αφορούν

(α) στα πνευματικά δικαιώματα της Διδακτορικής διατριβής μου με τίτλο «Μηχανισμοί της μυϊκής λειτουργίας: Η επίδραση της ουραιμίας στη βιοχημική κατάσταση και την ανοχή στο οξειδωτικό στρες»

(β) στη διαχείριση των ερευνητικών δεδομένων που θα συλλέξω στην πορεία εκπόνησής της:

1. Τα πνευματικά δικαιώματα του τόμου της μεταπτυχιακής ή διδακτορικής διατριβής που θα προκύψει θα ανήκουν σε μένα. Θα ακολουθήσω τις οδηγίες συγγραφής, εκτύπωσης και κατάθεσης αντιτύπων της διατριβής στα ανάλογα αποθετήρια (σε έντυπη ή/και σε ηλεκτρονική μορφή).
2. Η διαχείριση των δεδομένων της διατριβής ανήκει από κοινού σε εμένα και στον/στην κύριο επιβλέποντα -ουσα καθηγητή -τρια.
3. Οποιαδήποτε επιστημονική δημοσίευση ή ανακοίνωση (αναρτημένη ή προφορική), ή αναφορά που προέρχεται από το υλικό/δεδομένα της εργασίας αυτής θα γίνεται με συγγραφείς εμένα τον ίδιο, τον/την κύριο-α επιβλέποντα -ουσα ή/και άλλους ερευνητές (πχ μέλη της τριμελούς συμβουλευτικής επιτροπής, συνεργάτες κλπ), ανάλογα με τη συμβολή τους στην έρευνα και στη συγγραφή των ερευνητικών εργασιών.
4. Η σειρά των ονομάτων στις επιστημονικές δημοσιεύσεις ή επιστημονικές ανακοινώσεις θα αποφασίζεται από κοινού από εμένα και τον/την κύριο -α επιβλέποντα -ουσα της εργασίας, πριν αρχίσει η εκπόνησή της. Η απόφαση αυτή θα πιστοποιηθεί εγγράφως μεταξύ εμού και του/της κύριου-ας επιβλέποντος -ουσας.

**Τέλος, δηλώνω ότι γνωρίζω τους κανόνες περί δεοντολογίας και περί λογοκλοπής και πνευματικής ιδιοκτησίας και ότι θα τους τηρώ απαρέγκλιτα καθ' όλη τη διάρκεια της φοίτησης και κάλυψης των εκπαιδευτικών υποχρεώσεων μου που προκύπτουν από το ΠΜΣ/τμήμα και καθ' όλη τη διάρκεια των διαδικασιών δημοσίευσης που θα προκύψουν μετά την ολοκλήρωση των σπουδών μου.**

20/10/2012

Η δηλούσα

Κωνσταντίνα Πουλιανίτη

## APPENDIX B



### Εσωτερική Επιτροπή Δεοντολογίας

Τρίκαλα: 10/10/12

Αριθμ. Πρωτ.: 631

**Αίτηση Εξέτασης της πρότασης για διεξαγωγή Έρευνας με τίτλο:** «Μηχανισμοί της μυϊκής λειτουργίας: η επίδραση της ουραιμίας στην βιοχημική κατάσταση και την ανοχή στο οξειδωτικό στρες»

**Επιστημονικώς υπεύθυνος-η / επιβλέπων-ουσα:** Δρ. Χριστίνα Καρατζαφέρη

**Ιδιότητα:** Επίκουρη

Καθηγήτρια

**Ίδρυμα:** Τ.Ε.Φ.Α.Α

**Τμήμα:** Πανεπιστήμιο Θεσσαλίας

**Κύριος ερευνητής-τρια / φοιτητής-τρια:**

Κωνσταντίνα Πουλιανίτη **Πρόγραμμα**

**Σπουδών:** Απόκτηση διδακτορικού διπλώματος

**Ίδρυμα:** Τ.Ε.Φ.Α.Α

**Τμήμα:** Πανεπιστήμιο Θεσσαλίας

**Η προτεινόμενη έρευνα θα είναι:**

Ερευνητικό πρόγραμμα  Μεταπτυχιακή διατριβή  Διπλωματική εργασία

Διδακτορική έρευνα

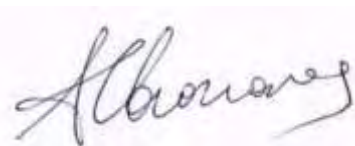
**Τηλ. επικοινωνίας:** 6975584990

**Email επικοινωνίας:** poulianiti.konstantina@yahoo.gr

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Η Εσωτερική Επιτροπή Δεοντολογίας του Τ.Ε.Φ.Α.Α., Πανεπιστημίου Θεσσαλίας μετά την υπ. Αριθμ. 2-2/10-10-2012 συνεδρίασή της εγκρίνει τη διεξαγωγή της προτεινόμενης έρευνας.

Ο Πρόεδρος της Εσωτερικής Επιτροπής Δεοντολογίας – ΤΕΦΑΑ



Τσιόκανος Αθανάσιος Αναπλ. Καθηγητής

## APPENDIX C

### Σύσταση τροφής κονίκλων

#### D30006 and D07122101 Low Phytoestrogen Rabbit Diet and Same With Lower Protein, Potassium, Calcium, Phosphorus, and Sodium

Product #	D30006		D07122101	
	gm%	kcal%	gm%	kcal%
Protein	17.8	22	8.9	11
Carbohydrate	55.0	68	63.6	79
Fat	3.5	10	3.5	10
Total		100		100
kcal/gm	3.23		3.21	
<b>Ingredient</b>	<b>gm</b>	<b>kcal</b>	<b>gm</b>	<b>kcal</b>
Casein, 30 Mesh	175	700	87.5	350
DL-Methionine	3	12	1.5	6
Corn Starch	390	1560	459	1836
MaltoDextrin 10	25	100	25	100
Sucrose	125	500	145	580
Cellulose, BW200	150	0	150	0
Inulin	25	0	25	0
Soybean Oil	35	315	35	315
Mineral Mix S30003	60	0	0	0
Mineral Mix S39102	0	0	60	0
Vitamin Mix V30002	10	40	10	40
Choline Bitartrate	2	0	2	0
Sodium Bicarbonate	0	0	4	0
<b>Total</b>	<b>1000</b>	<b>3227</b>	<b>1004</b>	<b>3227</b>
<b>gm%</b>				
Potassium	1.0		0.25	
Calcium	0.8		0.4	
Phosphorus	0.5		0.126	
Sodium	0.2		0.2	
Chloride	0.3		0.2	
Iron	0.01		0.01	

Formulated by E.A. Ulman, Ph.D., Research Diets, Inc., 7/9/01.  
Using purified ingredients, this diet matches Purina 5321. It is very low in phytoestrogens.

## APPENDIX D

<b>Biochemical and Hematological reference values for New Zealand rabbits</b>	
Total Cholesterol (mg/dl)	10-80
HDL Cholesterol (mg/dl)	15-30
LDL Cholesterol (mg/dl)	20-40
Triglycerides (mg/dl)	> 155
Glucose (mg/dl)	75-140
Bilirubin (mg/dl)	<1,16
Uric acid (mg/dl)	1-4.3
Leykocytes ( $10^3/\mu\text{L}$ )	5.1-13
Red Blood Cells ( $10^6/\text{mm}^3$ )	3.8-7.9
Hemoglobin (g/dl)	9.4-17.4
Hematocrit (%)	26.7-47.2
MCV ( $\text{mm}^3$ )	50-75
MCH (pg/cell)	18-24

References: (Dontas et al., 2011; Hewitt, Innes, Savory, & Wills, 1989; MediRabbit.com)



## APPENDIX E

<b>Redox status and Biochemical profile in a typical New Zealand rabbit (no intervention) analyzed in our laboratories.</b>		
	mixed muscle	blood
Total Protein (mg/ml)	5,43	68,415
Uric acid (mg/dl)	0,437	0,680
GSH ( $\mu\text{mol/gr}$ protein)	3,21	
GSSG ( $\mu\text{mol/gr}$ protein)	0,148	
Ratio (GSH/GSSG)	22	
GR Reductase (U/gr protein)	7,482	218,269
TAC ( $\mu\text{mol DPPH/ml}$ )	0,675	0,749
CAT (U/mg protein)	24,16	328,980
PC (nmol/mg protein)	0,167	0,625
TBARS (nmol/ml)	3,378	5,663
Total Cholesterol (mg/dl)	10,45	113,500
HDL (mg/dl)		57,850
LDL (mg/dl)		35,550
Triglycerides (mg/dl)	172	100,5
Glucose (mg/dl)		438,000
Bilirubin (mg/dl)		0,170