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The Selenoprotein T-mimetic (PSELT) exerts cardiometabolic protection in rat and mouse models of obesity and metabolic syndrome

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Riassunto

La sindrome metabolica (MetS) viene riconosciuta come un insieme di anomalie comprendenti obesità, dislipidemia, ridotta tolleranza al glucosio, insulino-resistenza, ipertensione e aumento del rischio di malattie cardiovascolari (*Cao et al., 2014*; *Della Vedova et al., 2016*). L'obesità, considerato il principale fattore di rischio coinvolto nella patogenesi della MetS, è una condizione clinica dovuta principalmente ad uno squilibrio tra apporto energetico e dispendio energetico (*Fraulob et al., 2010*). L'eccessiva deposizione lipidica che si verifica nell'obesità è associata a stress ossidativo, quest'ultimo è considerato un fattore critico coinvolto nella patogenesi di malattie metaboliche e nello sviluppo di comorbilità dipendenti dall'obesità (*Manna et al., 2015*; *Ogrodnik et al., 2019*). Un prolungato stato ossidativo determina sia danni alle biomolecole con disfunzioni cellulari e tissutali, che aumento dei livelli circolanti di glucosio e lipidi, in grado di modificare il metabolismo basale (*Rani et al., 2016*; *Marrocco et al., 2017*; *McMurray et al., 2016*). Diverse evidenze suggeriscono che uno dei meccanismi alla base delle malattie cardiovascolari sia rappresentato dalla sovrapproduzione di ROS (*Senoner & Dichtl, 2019*). Diversi studi hanno infatti mostrato il coinvolgimento dei ROS nell'ischemia miocardica acuta e nel danno da ischemia/riperfusione (I/R) (*Yellon & Hausenloy, 2007*; *Braunwald et al., 1985*). Inoltre, diversi fattori di rischio cardiovascolare, tra cui ipercolesterolemia, ipertensione arteriosa e iperglicemia, sono associati a una funzione endoteliale compromessa, quest'ultima è stata collegata ad obesità e al basso grado di infiammazione sistemica caratterizzante questa malattia metabolica (*Favero et al., 2014*; *Hadi et al., 2005*). Considerato il ruolo cruciale dello stress ossidativo nei disordini metabolici e nelle malattie cardiovascolari, è ipotizzabile che strategie volte a contrastare la formazione di ROS o ad aumentare le difese antiossidanti possano contribuire a prevenire e/o trattare alterazioni cardiache e manifestazioni sistemiche indotte dall'obesità (*Savini et al., 2013*; *D'Oria et al., 2020*). Nell'ambito delle difese antiossidanti endogene, le selenoproteine rappresentano una particolare classe di proteine in grado di incorporare il selenio, in forma organica sottoforma di selenocisteina (Sec). Questa famiglia di proteine è coinvolta in diversi processi biologici tanto che anomalie selettive a carico di questa classe di proteine possono essere spesso associate a malattie cardiovascolari, disturbi neurodegenerativi, disturbi immunitari ed endocrini, malattie del fegato e cancro (*Papp et al., 2007*). Ad oggi, tutte le selenoproteine attualmente note esercitano importanti attività di ossidoreduttasi attraverso il loro sito catalitico contenente Sec, e sono in grado di catalizzare reazioni redox basate sull'ossidazione di gruppi sulfidrilici e/o sulla riduzione di disolfuri (*Steinbrenner & Sies, 2009*). La selenoproteina T (SELENOT) è una selenoproteina residente nel reticolo

endoplasmatico, simile alla tioredossina (Trx), la cui ablazione genetica, nei topi, è associata ad un fenotipo letale nello stadio embrionale precoce (*Anouar et al., 2018*; *Hamieh et al., 2017*; *Pothion et al., 2020*). Diverse evidenze sperimentali hanno mostrato che questa selenoproteina, espressa ad alti livelli durante l'embriogenesi, risulta assente nei tessuti adulti ad eccezione dei tessuti endocrini, e viene riespressa o risulta essere upregolata in condizioni di stress ossidativo e del reticolo endoplasmico, infiammazione, esposizione a metalli pesanti e neurotossine (*Pothion et al., 2020*). Attraverso l'utilizzando di topi *knockout* condizionati per la SELENOT nelle cellule β-pancreatiche è stato dimostrato che la carenza di questa proteina provoca una riduzione nelle dimensioni delle isole pancreatiche e ridotta tolleranza al glucosio, indicando il suo coinvolgimento nella regolazione del metabolismo glucidico e nella sensibilità all'insulina (*Prevost et al., 2013*). Inoltre, è stato dimostrato che la SELENOT assente nel cuore di ratto adulto, risulta essere riespressa in seguito a danno miocardico da ischemia/riperfusione e in grado di mediare cardioprotezione (*Rocca et al., 2018*). Sebbene siano state accumulate numerose evidenze sperimentali che attestano il ruolo cruciale di SELENOT nel danno indotto dallo stress ossidativo in diversi contesti fisiopatologici (*Boukhzar et al., 2016*; *Rocca et al., 2018*), attualmente nulla è noto riguardo al ruolo di SELENOT in disordini metabolici quali obesità e sindrome metabolica. Pertanto, il presente progetto di dottorato ha avuto come obiettivi: *i)* investigare l'effetto protettivo a livello sistemico e cardiaco della somministrazione cronica di un peptide derivato da SELENOT (SELENOT $_{43\text{-}52}$), PSELT, che comprende il motivo redox (Cys-Val-Ser-Sec) della proteina *full length*, su modelli di ratto e topo con obesità e sindrome metabolica indotta dalla dieta; *ii)* valutare la capacità di PSELT di contrastare le tipiche complicanze dipendenti dall'obesità e dalla sindrome metabolica, come ridotta tolleranza al glucosio/insulino-resistenza e dismetabolismo lipidico, stress ossidativo e suscettibilità all'infarto del miocardio.

Nella prima parte del progetto è stato valutato l'effetto di PSELT in un modello di ratto con obesità indotta dal consumo di una dieta ipercalorica; a tale scopo, sono stati esaminati settimanalmente i parametri "antropometrici" e sono stati valutati a fine trattamento i parametri metabolici. In primo luogo, è stata registrata una tendenza verso la riduzione del peso e dell'indice di massa corporea nei ratti alimentati con dieta iperlipidica per 12 settimane e trattati con PSELT solo nelle ultime 4 settimane rispetto ai ratti alimentati con la sola dieta iperlipidica. La valutazione della glicemia a digiuno ha mostrato dei livelli significativamente ridotti nei ratti obesi trattati con PSELT rispetto ai ratti alimenti con la sola dieta iperlipidica. La curva da carico, eseguita nell'ultima settimana di dieta per valutare anomalie nel metabolismo del glucosio, ha indicato un miglioramento nella tolleranza al glucosio nei ratti obesi trattati con PSELT, corroborando il ruolo del peptide nel metabolismo glucidico e nella sensibilità all'insulina. Inoltre, l'analisi riguardante la deposizione lipidica a livello del tessuto

adiposo addominale, perirenale, epididimale e retroperitoneale, eseguita al termine del sacrificio, ha mostrato una riduzione significativa in tutti i distretti anatomici considerati nei ratti obesi trattati con il peptide. L'effetto di PSELT nelle alterazioni secondarie ad obesità e sindrome metabolica quali dislipidemia e disfunzione endoteliale, è stato valutato analizzando sezioni cardiache, epatiche e di aorta addominale mediante microscopia elettronica a trasmissione (TEM). Le analisi di microscopia hanno mostrato un accumulo citoplasmatico di lipidi e granuli di lipofuscina nei cuori dei ratti obesi, tuttavia la deposizione lipidica è risultata fortemente ridotta nei cuori dei ratti obesi trattati con PSELT. Parimenti, l'analisi ultrastrutturale eseguita su sezioni epatiche ha evidenziato un miglioramento della microarchitettura epatica e una riduzione della steatosi nel fegato dei ratti obesi trattati con il peptide, indicando che il peptide esercita un'azione citoprotettiva volta a contrastare l'accumulo ectopico di lipidi. E' ampiamente noto che l'obesità è associata a disfunzione endoteliale attraverso meccanismi indiretti con fattori di rischio cardiovascolari (*Engin 2017*). Per studiare il ruolo di PSELT a livello dell'endotelio dell'aorta addominale sono state condotte analisi di microscopia al TEM. I risultati hanno mostrato una convenzionale architettura delle cellule endoteliali dell'aorta nei ratti obesi trattati con il peptide, al contrario, è stata evidenziata la presenza di apoptosi/necrosi a livello dell'endotelio con distacco delle cellule dal tessuto connettivo nei campioni provenienti dai ratti obesi. L'azione cardioprotettiva del peptide è stata studiata a fine trattamento quantificando marker specifici di danno cardiaco e valutando la suscettibilità all'infarto miocardico in un modello di cuore di ratto isolato e perfuso secondo metodica Langendorff e sottoposto a protocollo di ischemia/riperfusione (*Rocca et al., 2018*). La valutazione dei livelli di peptide natriuretico di tipo B (BNP), noto marker di insufficienza cardiaca, ha mostrato una riduzione significativa nei ventricoli sinistri dei ratti obesi trattati con PSELT rispetto ai ventricoli provenienti da ratti alimentati con la sola dieta iperlipidica. Inoltre, la somministrazione cronica di PSELT 15 µg/kg/die nelle ultime 4 settimane di dieta ipercalorica, ha esercitato un'azione cardioprotettiva, come evidenziato dal miglioramento della funzione sistolica al basale, dal significativo recupero post-ischemico della contrattilità esaminata in termini di pressione ventricolare sinistra (dLVP) e dalla riduzione dell'area d'infarto (IS); inoltre, nessuna variazione della contrattura cardiaca è stata registrata durante la riperfusione (indicata in termini di pressione ventricolare sinistra endo-diastolica, LVEDP).

L'azione citoprotettiva di PSELT e il contributo della selenoproteina T endogena nella protezione indotta dal peptide esogeno contro lo stress ossidativo *in vitro*, è stata analizzata utilizzando cardiomiociti embrionali di ratto (H9c2) esposti a perossido di idrogeno (H_2O_2). *In primis* è stata valutata la vitalità cellulare in cardiomiociti pretrattati con PSELT e poi esposti a danno ossidativo diretto da H_2O_2. Analisi di MTT (3-4,5-dimethylthiazol-2,5-diphenyl tetrazonium bromide) hanno mostrato un incremento significativo dose-dipendente

della vitalità cellulare nelle cellule H9c2 pretrattate con il peptide e poi esposte ad H_2O_2. Al contrario, un peptide di controllo privo del dominio redox (indicato come *Inert* PSELT), in cui il residuo Sec è stato sostituito con un residuo di Ser, non ha determinato alcuna variazione significativa in termini di vitalità cellulare in seguito ad esposizione a perossido di idrogeno. Analisi di western blot eseguite su cardiomiociti esposti a concentrazioni crescenti di PSELT e trattate con perossido di idrogeno, hanno mostrato che la citoprotezione è accompagnata da un aumento dell'espressione della SELENOT nelle cellule pretrattate con il peptide ed esposte ad H_2O_2. Per discriminare il contributo della proteina endogena nella protezione conferita dal peptide esogeno, sono stati eseguiti saggi di silenziamento genico mediante l'utilizzo di small interfering RNA (siRNA). L'efficienza del *knockdown*, valutata mediante analisi di immunoblot, ha mostrato una marcata riduzione della SELENOT nelle cellule transfettate con il siRNA rispetto alle cellule transfettate con il controllo negativo (si-NC). Successivamente, è stata valutata la vitalità cellulare in cellule silenziate per la SELENOT, pretrattate con PSELT e poi esposte a danno ossidativo con perossido di idrogeno. Le analisi eseguite mediante saggio MTT hanno rivelato una significativa riduzione della vitalità nelle cellule silenziate per SELENOT rispetto alle cellule transfettate con il controllo negativo. Inoltre, il pretrattamento con PSELT, nelle cellule silenziate per la proteina endogena, ha determinato un incremento della vitalità cellulare nei cardiomiociti anche esposti ad H_2O_2.

Per valutare l'azione di PSELT in un modello di lipotossicità *in vitro*, è stato utilizzato il palmitato, noto induttore di stress e danno lipotossico a livello cardiaco (*Kong & Rabkin, 2000*). Analisi di MTT hanno evidenziato una ridotta vitalità cellulare in risposta al trattamento con dosi crescenti di palmitato nei cardiomiociti H9c2. Analisi successive sulla vitalità cellulare, condotte utilizzando la prima dose utile di palmitato in grado di determinare morte cellulare a livello dei cardiomiociti e dosi crescenti di PSELT, hanno rivelato un significativo incremento della vitalità cellulare nelle cellule trattate in combinazione con palmitato e PSELT a partire dalla dose di 5 nM del peptide. La colorazione Oil Red O è stata utilizzata per verificare l'accumulo di lipidi intracellulari indotto dal palmitato e per indagare la potenziale azione del PSELT nel contrastare la deposizione lipidica a livello cellulare. Dati relativi ad analisi microscopica hanno mostrato una marcata riduzione in termini di deposizione di gocce lipidiche intracellulari nei cardiomiociti trattati con PSELT.

La seconda parte del progetto ha avuto l'obiettivo di valutare l'azione di PSELT in disturbi associati al comportamento alimentare, in particolare relativi allo stato d'ansia e alla ridotta capacità locomotoria, in un modello murino con obesità indotta dalla dieta. A tal proposito, sono state effettuate settimanalmente misurazioni relative al peso corporeo e al *food intake*, ed è stata valutata nelle ultime 4 settimane di dieta la glicemia a digiuno. La valutazione del peso corporeo ha mostrato un decremento significativo nei topi alimentati con dieta

ipercalorica e trattati con PSELT rispetto ai topi alimentati con la sola dieta ipercalorica. E' stato inoltre mostrato che la riduzione del peso corporeo, nei topi obesi trattati con PSELT, era attribuibile ad una riduzione del cibo ingerito; tuttavia, nessuna variazione significativa in termini di composizione corporea è stata osservata all'interno dei gruppi sperimentali alimentati con dieta ipercalorica. Le misurazioni della glicemia a digiuno hanno mostrato una riduzione significativa nei topi obesi trattati con PSELT sia a partire dalla prima settimana di dieta che nelle ultime 8 settimane, corroborando, anche in questo caso, l'azione del PSELT nel metabolismo del glucosio. Considerato che l'obesità viene spesso associata a disturbi quali ansia e depressione, e che lo stato ansioso è un tratto distintivo dei pazienti obesi, è stata valutata l'azione di PSELT nella regolazione dello stato d'ansia, mediante Open field. Le analisi condotte hanno mostrato un trend verso la riduzione del fenotipo ansioso nei topi obesi trattati con PSELT a partire dalla prima settimana di dieta ipercalorica rispetto ai topi obesi, tuttavia nessun dato statisticamente significativo è stato riportato. Inoltre, l'analisi relativa all'attività locomotoria non ha registrato alcuna variazione all'interno dei gruppi sperimentali, dunque la capacità locomotoria non è stata intaccata dal consumo della dieta ipercalorica.

I presenti risultati evidenziano il PSELT come potenziale modulatore cardio-metabolico e come un possibile agente farmacologico in grado di contrastare le comorbidità secondarie ad obesità e sindrome metabolica.

Complessivamente, i dati riguardanti l'azione protettiva di PSELT in modelli di ratto/topo con obesità indotta dalla dieta e in modelli *in vitro* di stress ossidativo e lipotossicità forniscono nuove informazioni sull'influenza del peptide derivato dalla SELENOT, PSELT, nel metabolismo sistemico e cardiaco in condizioni dismetaboliche e di stress ossidativo, nonchè in presenza di comorbidità secondarie ad obesità. Queste osservazioni aprono la strada a studi futuri volti ad indagare la possibile rilevanza clinica di PSELT nell'ambito delle malattie metaboliche e cardiometaboliche.

Résumé

Le syndrome métabolique (MetS) est reconnu comme un ensemble d'anomalies comprenant l'obésité, la dyslipidémie, l'intolérance réduite au glucose, la résistance à l'insuline, l'hypertension et un risque accru de maladies cardiovasculaires (*Cao et al., 2014* ; *Della Vedova et al., 2016*). L'obésité, considérée comme le principal facteur de risque impliqué dans la pathogenèse du MetS, est un état clinique principalement dû à un déséquilibre entre l'apport énergétique et la dépense énergétique (*Fraulob et al., 2010*). Le dépôt excessif de lipides qui se produit dans l'obésité est associé au stress oxydatif, ce dernier étant considéré comme un facteur critique impliqué dans la pathogenèse des maladies métaboliques et le développement de comorbidités dépendantes de l'obésité (*Manna et al., 2015* ; *Ogrodnik et al., 2019*). Un état oxydatif prolongé entraîne à la fois des dommages aux biomolécules avec un dysfonctionnement cellulaire et tissulaire et une augmentation des taux circulants de glucose et de lipides, ce qui peut altérer le métabolisme basal (*Rani et al., 2016* ; *Marrocco et al., 2017* ; *McMurray et al., 2016*). Plusieurs observations suggèrent que l'un des mécanismes à l'origine des maladies cardiovasculaires est représenté par la surproduction de ROS (*Senoner & Dichtl, 2019*). En effet, plusieurs études ont montré l'implication des ROS dans l'ischémie aiguë du myocarde et les lésions d'ischémie/reperfusion (I/R) (*Yellon & Hausenloy, 2007* ; *Braunwald et al., 1985*). Par ailleurs, plusieurs facteurs de risque cardiovasculaire, dont l'hypercholestérolémie, l'hypertension et l'hyperglycémie, sont associés à une altération de la fonction endothéliale, cette dernière ayant été liée à l'obésité et au faible degré d'inflammation systémique caractérisant cette maladie métabolique (*Favero et al., 2014* ; *Hadi et al., 2005*). Étant donné le rôle crucial du stress oxydatif dans les troubles métaboliques et les maladies cardiovasculaires, il est tout à fait possible que les stratégies visant à contrer la formation de ROS ou à augmenter les défenses antioxydantes puissent contribuer à prévenir et/ou à traiter les altérations cardiaques et les manifestations systémiques induites par l'obésité (*Savini et al., 2013* ; *D'Oria et al., 2020*). Parmi les défenses antioxydantes endogènes, les sélénoprotéines représentent une classe particulière de protéines capables d'incorporer le sélénium sous forme organique en tant que sélénocystéine (Sec). Cette famille de protéines est impliquée dans différents processus biologiques, et des anomalies dans cette classe de protéines peuvent souvent être associées à des maladies cardiovasculaires, des troubles neurodégénératifs, des troubles immunitaires et endocriniens, des maladies du foie et des cancers (*Papp et al., 2007*). À ce jour, toutes les sélénoprotéines actuellement connues exercent des activités d'oxydoréduction importantes par l'intermédiaire de leur site catalytique contenant la Sec, et sont capables de catalyser des réactions d'oxydation des groupes sulphydryles et/ou la réduction des disulfures (*Steinbrenner & Sies, 2009*). La sélénoprotéine T (SELENOT) est une

sélénoprotéine résidant dans le réticulum endoplasmique, ressemblant à la thiorédoxine (Trx), dont l'ablation génétique, chez la souris, est associée à un phénotype létal au stade embryonnaire précoce (*Anouar et al., 2018* ; *Hamieh et al., 2017* ; *Pothion et al., 2020*). Plusieurs preuves expérimentales ont montré que cette sélénoprotéine, exprimée à des niveaux élevés pendant l'embryogenèse, est absente des tissus adultes à l'exception des tissus endocriniens, et est stimulée ou induite dans des conditions de stress oxydatif et de stress du réticulum endoplasmique, d'inflammation, d'exposition aux métaux lourds et aux neurotoxines (*Pothion et al., 2020*). Grâce à l'utilisation de souris knockout conditionnelles pour la SELENOT dans les cellules β-pancréatiques, il a été montré que la déficience de cette protéine entraîne une réduction de la taille des îlots pancréatiques et une altération de la tolérance au glucose, indiquant son implication dans la régulation du métabolisme du glucose et de la sensibilité à l'insuline (*Prevost et al., 2013*). En outre, il a été démontré que SELENOT, absent dans le cœur du rat adulte, est réexprimé après des dommages myocardiques ischémiques après reperfusion et est capable d'assurer une cardioprotection (*Rocca et al., 2018*). Bien que de nombreuses preuves expérimentales aient été accumulées attestant du rôle crucial de la SELENOT dans les dommages induits par le stress oxydatif dans différents contextes physiopathologiques (*Boukhzar et al., 2016* ; *Rocca et al., 2018*), on ne sait actuellement rien du rôle de la SELENOT dans les troubles métaboliques tels que l'obésité et le syndrome métabolique. Par conséquent, les objectifs du présent projet de thèse étaient les suivants: i) étudier l'effet protecteur systémique et cardiaque de l'administration chronique d'un peptide dérivé de la SELENOT ($SELENOT_{43-52}$), PSELT, comprenant le motif redox (Cys-Val-Ser-Sec) de la protéine, sur des modèles de rats et de souris souffrant d'obésité et de syndrome métabolique induit par l'alimentation; ii) évaluer la capacité du PSELT à contrer les complications typiques de l'obésité et du syndrome métabolique, telles que l'intolérance au glucose/résistance à l'insuline et le dysmétabolisme lipidique, le stress oxydatif et la susceptibilité à l'infarctus du myocarde.

Dans la première partie du projet, l'effet du PSELT a été évalué dans un modèle de rat souffrant d'obésité induite par la consommation d'un régime hypercalorique ; pour cela, les paramètres "anthropométriques" ont été examinés chaque semaine et les paramètres métaboliques ont été évalués à la fin du traitement. Tout d'abord, une tendance à la réduction du poids et de l'indice de masse corporelle a été enregistré chez les rats nourris avec le régime hyperlipidique pendant 12 semaines et traités avec PSELT uniquement au cours des 4 dernières semaines, par rapport aux rats nourris avec le régime hyperlipidique seul. L'évaluation de la glycémie à jeun a montré des niveaux significativement réduits chez les rats obèses traités avec PSELT par rapport aux rats nourris avec le régime hyperlipidique seul. La courbe de charge, réalisée au cours de la dernière semaine du régime pour évaluer les anomalies du métabolisme du glucose, a indiqué une amélioration de la tolérance au glucose chez les rats obèses traités par PSELT, ce

qui montre un effet du peptide dans le métabolisme du glucose et la sensibilité à l'insuline. En outre, l'analyse du dépôt de lipides dans le tissu adipeux abdominal, épididymaire et rétropéritonéal, effectuée après le sacrifice de l'animal, a montré une réduction significative dans tous les localisations considérées chez les rats obèses traités avec le peptide. L'effet du PSELT sur les changements secondaires à l'obésité et au syndrome métabolique, tels que la dyslipidémie et le dysfonctionnement endothélial, a été évalué en analysant des coupes de cœur, du foie et de l'aorte abdominale à l'aide de la microscopie électronique à transmission (MET). L'analyse microscopique a montré une accumulation cytoplasmique de lipides et de granules de lipofuscine dans les cœurs des rats obèses, alors que les dépôts de lipides étaient fortement réduits dans le cœur des rats obèses traités par le PSELT. De même, l'analyse ultrastructurale de sections de foie a montré une amélioration de la microarchitecture hépatique et une réduction de la stéatose dans le foie de rats obèses traités avec le peptide, ce qui indique que le peptide exerce une action cytoprotective pour contrer l'accumulation ectopique de lipides. Il est connu que l'obésité est associée à la dysfonction endothéliale par des mécanismes indirects impliquant les facteurs de risque cardiovasculaire (*Engin 2017*). Pour étudier le rôle de PSELT au niveau de l'endothélium de l'aorte abdominale, des analyses par MET ont été réalisées. Les résultats ont montré une architecture conventionnelle des cellules endothéliales de l'aorte chez les rats obèses traités avec le peptide, alors que dans les échantillons des rats obèses la présence d'apoptose/nécrose au niveau de l'endothélium avec détachement des cellules du tissu conjonctif a été mise en évidence. L'action cardioprotectrice du peptide a été étudiée à la fin du traitement en quantifiant des marqueurs spécifiques de dommages cardiaques et en évaluant la susceptibilité à l'infarctus du myocarde dans un modèle de cœur de rat isolé et perfusé selon la méthode de Langendorff et soumis à un protocole d'ischémie/reperfusion (*Rocca et al., 2018*). L'évaluation des niveaux de peptide natriurétique de type B (BNP), un marqueur connu de l'insuffisance cardiaque, a montré une réduction significative dans les ventricules gauches des rats obèses traités par PSELT par rapport aux ventricules des rats nourris avec le seul régime hyperlipidique. En outre, l'administration chronique de PSELT 15 µg/kg/jour pendant les 4 dernières semaines du régime hyperlipidique a permis de montrer son action cardioprotectrice, comme en témoignent l'amélioration de la fonction systolique au départ, la récupération post-ischémique significative de la contractilité examinée en termes de pression ventriculaire gauche (dLVP) et la réduction de la zone d'infarctus (IS). En outre, aucune modification de la contractilité cardiaque n'a été enregistrée pendant la reperfusion (indiquée en termes de pression ventriculaire gauche endo-diastolique, LVEDP).

L'action cytoprotectrice du PSELT et la contribution de la SELENOT endogène à la protection induite par le peptide exogène contre le stress oxydatif *in vitro* ont été étudiées en utilisant des cardiomyocytes embryonnaires de rat (H9c2) exposés au peroxyde d'hydrogène (H_2O_2). Tout

d'abord, la viabilité cellulaire a été évaluée dans les cardiomyocytes prétraités avec le PSELT et ensuite exposés à des dommages oxydatifs directs par H_2O_2. Les analyses de MTT ont montré une augmentation significative et dose-dépendante de la viabilité cellulaire dans les cellules H9c2 prétraitées avec le peptide et ensuite exposées à H_2O_2. En revanche, un peptide témoin dépourvu du domaine redox (appelé PSELT inerte), dans lequel le résidu Sec a été remplacé par un résidu Ser, n'a montré aucun changement significatif de la viabilité cellulaire après exposition au peroxyde d'hydrogène. Des analyses Western blot réalisées sur des cardiomyocytes exposés à des concentrations croissantes de PSELT et traités au peroxyde d'hydrogène ont montré que la cytoprotection s'accompagne d'une augmentation de l'expression de SELENOT dans les cellules prétraitées par le peptide et exposées au H_2O_2. Afin de distinguer la contribution de la protéine endogène dans la protection conférée par le peptide exogène, des essais de silençage de gènes utilisant des petits RNA interférents (siRNA) ont été réalisés. L'efficacité du knockdown, évaluée par analyse immunoblot, a montré une réduction marquée de SELENOT dans les cellules transfectées avec le siRNA par rapport aux cellules transfectées avec le contrôle négatif (si-NC). Par la suite, la viabilité cellulaire a été évaluée dans les cellules sous-exprimant la SELENOT, prétraitées avec le PSELT et ensuite exposées à des dommages oxydatifs avec du peroxyde d'hydrogène. L'analyse par le test MTT a révélé une réduction significative de la viabilité des cellules privées de SELENOT par rapport aux cellules transfectées avec le contrôle négatif. En outre, le prétraitement avec PSELT, dans des cellules sous-exprimant la protéine endogène, a entraîné une augmentation de la viabilité cellulaire dans les cardiomyocytes également exposés à H_2O_2.

Pour évaluer l'action du PSELT dans un modèle de lipotoxicité *in vitro*, le palmitate, un inducteur connu de stress cardiaque et de dommages lipotoxiques, a été utilisé (Kong & Rabkin, 2000). Les analyses MTT ont montré une viabilité cellulaire réduite en réponse au traitement avec des doses croissantes de palmitate dans les cardiomyocytes H9c2. Des analyses ultérieures de viabilité cellulaire, utilisant la première dose utile de palmitate pour provoquer la mort cellulaire dans les cardiomyocytes et des doses croissantes de PSELT, ont révélé une augmentation significative de la viabilité cellulaire dans les cellules traitées avec du palmitate et du PSELT à partir d'une dose de 5 nM du peptide. La coloration Oil Red O a été utilisée pour vérifier l'accumulation de lipides intracellulaires induite par le palmitate et pour étudier l'action potentielle du PSELT dans la lutte contre le dépôt de lipides cellulaires. Les données d'analyse microscopique ont montré une réduction marquée du dépôt de gouttelettes lipidiques intracellulaires dans les cardiomyocytes traités par PSELT.

La deuxième partie du projet visait à évaluer l'action de PSELT dans les troubles liés à l'alimentation, notamment liés à l'anxiété et à la réduction de la capacité locomotrice, dans un modèle de souris avec obésité induite par le régime alimentaire. Dans ce but, des mesures

hebdomadaires du poids corporel et de la prise alimentaire ont été effectuées, et la glycémie à jeun a été évaluée pendant les 4 dernières semaines du régime. L'évaluation du poids corporel a montré une diminution significative chez les souris nourries avec le régime hypercalorique et traitées avec PSELT par rapport aux souris nourries avec le régime hypercalorique seul. Il a également été démontré que la réduction du poids corporel chez les souris obèses traitées avec le PSELT était due à une réduction de la prise alimentaire ; cependant, aucun changement significatif de la composition corporelle n'a été observé dans les groupes expérimentaux nourris avec le régime hypercalorique. Les mesures de la glycémie à jeun ont montré une réduction significative chez les souris obèses traitées par PSELT à la fois dès la première semaine du régime et au cours des 8 dernières semaines, ce qui corrobore à nouveau l'effet de PSELT sur le métabolisme du glucose. Étant donné que l'obésité est souvent associée à des troubles tels que l'anxiété et la dépression, et que l'anxiété est une caractéristique des patients obèses, l'effet du PSELT dans la régulation de l'état d'anxiété a été évalué en *Open field*. Les analyses ont montré une tendance à la réduction du phénotype d'anxiété chez les souris obèses traitées par PSELT dès la première semaine du régime hypercalorique par rapport aux souris obèses, mais aucune donnée statistiquement significative n'a été observé. De plus, l'analyse de l'activité locomotrice n'a montré aucune variation au sein des groupes expérimentaux, la capacité locomotrice n'a donc pas été affectée par la consommation du régime hypercalorique.

Les résultats actuels mettent en évidence le PSELT comme un modulateur cardio-métabolique potentiel et comme un agent pharmacologique possible capable de contrer les comorbidités secondaires à l'obésité et au syndrome métabolique.

Dans l'ensemble, les données concernant l'action protectrice du PSELT dans des modèles rat/souris d'obésité induite par l'alimentation et des modèles *in vitro* de stress oxydatif et de lipotoxicité fournissent de nouvelles informations sur l'influence du peptide dérivé de la SELENOT, le PSELT, sur le métabolisme systémique et cardiaque dans des conditions de dysmétabolisme et de stress oxydatif, ainsi qu'en présence de comorbidités secondaires à l'obésité. Ces observations ouvrent la voie à de futures études visant à examiner l'éventuelle pertinence clinique du PSELT dans les maladies métaboliques et cardiométaboliques.

Summary

Metabolic syndrome (MetS) is recognized as a cluster of abnormalities including obesity, dyslipidemia, impaired glucose tolerance, insulin resistance, hypertension and increased risk of cardiovascular disease (*Cao et al., 2014*; *Della Vedova et al., 2016*). Obesity, considered the main risk factor involved in the pathogenesis of MetS, is a clinical condition mainly due to an imbalance between energy intake and energy expenditure (*Fraulob et al., 2010*). The excessive lipid deposition occurs in obesity is associated with oxidative stress, the latter is considered a critical factor involved in the pathogenesis of metabolic diseases and in the development of obesity-dependent comorbidities (*Manna et al., 2015*; *Ogrodnik et al., 2019*). A prolonged oxidative state inevitably causes both damage to biomolecules with cell and tissue dysfunctions, and an increase in circulating levels of glucose and lipids, capable of modifying the basal metabolism *(Rani et al., 2016*; *Marrocco et al., 2017*; *McMurray et al., 2016*). Several evidence suggests that one of the mechanisms underlying cardiovascular disease is represented by ROS overproduction (*Senoner & Dichtl, 2019*). Several studies have been shown the involvement of ROS in acute myocardial ischemia and in ischemia/reperfusion (I/R) injury (*Yellon & Hausenloy, 2007*; *Braunwald et al., 1985*). Furthermore, several cardiovascular risk factors, including hypercholesterolemia, arterial hypertension and hyperglycemia, are associated with impaired endothelial function, the latter is linked to obesity and the low degree of systemic inflammation characterizing this metabolic disorder (*Favero et al., 2014*; *Hadi et al., 2005).* Considering the crucial role of oxidative stress in metabolic disorders and cardiovascular diseases, it is conceivable that strategies aimed to counteract ROS generation or increasing antioxidant defenses may help to prevent and/or treat cardiac alterations and systemic manifestations induced by obesity (*Savini et al., 2013*; *D'Oria et al., 2020*). As part of the endogenous antioxidant defenses, selenoproteins are considered a particular class of proteins incorporating selenium, in organic form as selenocysteine (Sec). This family of proteins is involved in various biological processes, extent that selective anomalies in this class of proteins can often be associated with cardiovascular diseases, neurodegenerative disorders, immune and endocrine disorders, liver disease and cancer (*Papp et al., 2007*). To date, all currently known selenoproteins exert important oxidoreductase activities through their catalytic site containing Sec, and are able to catalyze redox reactions based on the oxidation of sulfhydryl groups and/or on the reduction of disulfides (*Steinbrenner & Sies, 2009*). Selenoprotein T (SELENOT) is an endoplasmic reticulum-resident selenoprotein, similar to thioredoxin (Trx), whose genetic ablation, in mice, is associated with a lethal phenotype in the early embryonic stage (*Anouar et al., 2018*; *Hamieh et al., 2017; Pothion et al., 2020*). Several experimental evidence has shown that this selenoprotein, expressed at high levels during embryogenesis, is absent in adult tissues except

in endocrine tissues, and is re-expressed or is found to be upregulated under conditions of oxidative stress and endoplasmic reticulum, inflammation, exposure to heavy metals and neurotoxins (*Pothion et al., 2020*). By using SELENOT-conditioned *knockout* mice in β-pancreatic cells it has been demonstrated that the deficiency of this protein results in reduced pancreatic island size and reduced glucose tolerance, indicating its involvement in the regulation of glucose metabolism and insulin sensitivity (*Prevost et al., 2013*). Although numerous experimental evidences have been accumulated attesting the crucial role of SELENOT in oxidative stress-induced damage in different pathophysiological contexts (*Boukhzar et al., 2016*; *Rocca et al., 2018*), currently nothing is known regarding the role of SELENOT in metabolic disorders such as obesity and metabolic syndrome. Therefore, the present PhD project had as main goals: *i)* to investigate the protective effect at systemic and cardiac level, of chronic administration of SELENOT-derived peptide ($SELENOT_{43-52}$), PSELT, which includes the redox motif (Cys- Val-Ser-Sec) of the full length protein, in rat and mouse models with obesity and diet-induced metabolic syndrome; *ii)* to evaluate the ability of PSELT to counteract the typical complications related to obesity and metabolic syndrome, such as impaired glucose/insulin resistance and lipid dysmetabolism, oxidative stress and susceptibility to myocardial infarction.

In the first part of the project, the effect of PSELT in a rat model with obesity induced by the consumption of a high-calorie diet was evaluated; for this purpose, the "anthropometric" parameters were examined weekly, and the metabolic parameters were evaluated at the end of the treatment. First, there was a trend towards the reduction of weight and body mass index (BMI) in rats fed hyperlipidic diet for 12 weeks and treated with PSELT only in the last 4 weeks compared to rats fed hyperlipid diet alone. The evaluation of fasting glycemia showed significantly reduced levels in obese rats treated with PSELT compared to rats fed hyperlipidic diet alone. The glucose concentration curve, performed in the last week of diet, to assess abnormalities in glucose metabolism, indicated an improvement in glucose tolerance in obese rats treated with PSELT, corroborating the role of the peptide in glucose metabolism and insulin sensitivity. In addition, the analysis concerning lipid deposition in the abdominal, perirenal, epididymal and retroperitoneal adipose tissue, performed at the end of the sacrifice, showed a significant reduction in all the anatomical districts considered in the obese rats treated with the peptide. The effect of PSELT in secondary alterations to obesity and metabolic syndrome such as dyslipidemia and endothelial dysfunction was evaluated by analyzing cardiac sections, liver, and abdominal aorta using transmission electron microscopy (TEM). Microscopy analysis showed cytoplasmic accumulation of lipids and lipofuscin granules in the hearts of the obese rats, however lipid deposition was strongly reduced in the hearts of the obese rats treated with PSELT. Likewise, the ultrastructural analysis performed

on liver sections showed an improvement in hepatic microarchitecture and a reduction in steatosis in the liver of obese rats treated with the peptide, indicating that the peptide exerts a cytoprotective action aimed to counteract the ectopic lipids accumulation. It is widely known that obesity is associated with endothelial dysfunction through indirect mechanisms with cardiovascular risk factors (*Engin 2017*). Transmission electron microscopy analysis were conducted to study the role of PSELT at the endothelium level of the abdominal aorta. The results showed a conventional architecture of the aortic endothelial cells in obese rats treated with the peptide, on the contrary, the presence of apoptosis/necrosis at the level of the endothelium with detachment of the cells from the connective tissue, in the samples from the obese rats was highlighted. The cardioprotective action of the peptide was studied at the end of the treatment by quantifying specific markers of cardiac damage and evaluating the susceptibility to myocardial infarction in an isolated and perfused rat heart model according to the Langendorff method and subjected to an ischemia/reperfusion protocol (*Rocca et al., 2018*). The assessment of natriuretic peptide type B (BNP) levels, as prognostic marker of heart failure, showed a significant reduction in the left ventricles of obese rats treated with PSELT compared to the ventricles of obese rats alone. Furthermore, the chronic administration of PSELT 15 µg/kg/day in the last 4 weeks of a high-calorie diet, exerted a cardioprotective action, as evidenced by the improvement of the systolic function at baseline, by the significant post-ischemic recovery of the contractility examined in terms of left ventricular pressure (dLVP) and reduction of the infarct area (IS); furthermore, no change in cardiac contracture was recorded during reperfusion in both experimental groups treated with the peptide (indicated in terms of left ventricular end-diastolic pressure, LVEDP).

The cytoprotective action of PSELT and the contribution of endogenous selenoprotein T in the protection induced by the exogenous peptide against oxidative stress *in vitro*, was analyzed using rat embryonic cardiomyocytes (H9c2) exposed to hydrogen peroxide (H_2O_2). First, cell viability was assessed in cardiomyocytes pretreated with PSELT and then exposed to direct oxidative damage by H_2O_2. MTT analysis showed a significant increase in dose-dependent manner in term of cell viability in H9c2 pretreated with the peptide and then exposed to H_2O_2. On the contrary, a control peptide lacking the redox motif (referred to as Inert PSELT), within which the Sec residue was replaced with a Ser residue, did not result in any significant change in terms of cell viability following exposure to hydrogen peroxide. Western blot analysis performed on cardiomyocytes exposed to increasing concentrations of PSELT and treated with hydrogen peroxide, showed that cytoprotection is accompanied by an increase in the expression of SELENOT in cells pretreated with the peptide and exposed to H_2O_2. To discriminate the contribution of the endogenous protein in the protection conferred by the exogenous peptide, gene silencing assays were performed using small interfering RNA

(siRNA). The *knockdown* efficiency, evaluated by immunoblot analysis, showed a marked reduction of SELENOT in the cells transfected with siRNA compared to cells transfected with the negative control (si-NC). Subsequently, cell viability was assessed in cells silencing for SELENOT, pretreated with PSELT and then exposed to oxidative damage with hydrogen peroxide. Analysis performed by MTT assay revealed a significant reduction in viability in SELENOT-silenced cells compared to cells transfected with the negative control. Furthermore, the pretreatment with PSELT, in the cells silenced for the endogenous protein, resulted in an increase in cell viability in cardiomyocytes also exposed to H_2O_2.

To evaluate the action of PSELT in an *in vitro* model of lipotoxicity, palmitate, known inducer of stress and lipotoxic damage in the heart, was used (*Kong & Rabkin, 2000*). MTT analysis revealed decreased cell viability in response to treatment with increasing concentration of palmitate in H9c2 cardiomyocytes. Subsequent cell viability analysis, using the first useful dose of palmitate capable to provoke cell death at cardiomyocytes level, and increasing doses of PSELT, displayed a significant increase in cell viability in palmitate and PSELT-treated cells starting from the dose of 5 nM of the peptide. The Oil Red O staining was used to verify the accumulation of intracellular lipids induced by palmitate and to investigate the potential action of PSELT in counteracting lipid deposition at cellular level. Data from microscopic analysis showed a marked reduction in terms of deposition of intracellular lipid droplets in PSELT-treated H9c2 cardiomyocytes.

The second part of the project aimed to evaluate the action of PSELT in disorders associated with eating behaviour, in particular related to anxiety and reduced locomotor activity, in a mouse model with diet-induced obesity. In this regard, weekly measurements regarding body weight and food intake were carried out, and fasting glycaemia was assessed in the last 4 weeks of the diet. Body weight assessment showed a significant decrease in mice fed high calorie diet and treated with PSELT compared to mice fed high calorie diet alone. It was also shown that the reduction in body weight, in obese mice treated with PSELT, was attributable to a reduction in the food intake; however, no significant changes in terms of body composition were observed within the experimental groups fed with a high-calorie diet treated with or without PSELT. Fasting blood glucose measurements showed a significant reduction in obese mice treated with PSELT both from the first week of the diet and in the last 8 weeks, corroborating the action of PSELT in glucose metabolism.

Considering that obesity is often associated with disorders such as anxiety and depression, and that the anxious state is a distinctive trait of obese patients (*Ogrodnik et al., 2019 and references therein*), the action of PSELT on anxiety-like phenotype was evaluated by Open Field. The analysis conducted showed a trend towards the reduction of the anxious phenotype

in obese mice treated with PSELT starting from the first week of high calorie diet compared to obese mice, however no statistically significant difference was reported. In addition, the analysis related to locomotor activity did not record any change within the experimental groups, so the locomotor capacity was not affected by the consumption of the high-calorie diet.

The present results highlight PSELT as a potential cardio-metabolic modulator and as a possible pharmacological agent capable to counteract comorbidities secondary to obesity and metabolic syndrome.

Overall, the data regarding the protective action of PSELT in animal models with diet-induced obesity and *in vitro* models of oxidative stress and lipotoxicity provide new insights into the influence of the peptide derived from SELENOT, PSELT, on systemic and cardiac metabolism under dismetabolic and oxidant stressful conditions, as well as in the presence of comorbidities secondary to obesity. These observations pave the way for future studies aimed at investigating the possible clinical relevance of PSELT in the context of metabolic and cardiometabolic diseases.

Introduction

1. Obesity and Metabolic syndrome

1.1. Pathophysiology of obesity

Obesity is a complex chronic disease characterized by an excessive weight gain due to perturbed energy homeostasis regulation producing an imbalance between energy intake and energy expenditure (*Gadde et al., 2018*). This pathological condition represents one of the most common health problems with a constantly increasing prevalence in present society. A recent study that analysed the prevalence of obesity from 1980 to 2015 in 195 countries, revealed that approximately 603.7 million adults were obese, with an overall prevalence of 12% (*Afshin et al., 2017*). Currently, one of the most used parameters to establish overweight is the body mass index (BMI), which expresses the ratio between the weight in kilograms and the height in m^2. According to the BMI evaluation, population-based studies classified individuals with BMI between 18.5–24.9 kg/m^2 as normal-weight and BMI 25.0–29.9 kg/m^2 as overweight. Individuals presenting obesity can be further divided within subcategories: class 1 (30.0–34.9 kg/m^2), class 2 (35.0–39.9 kg/m^2) and class 3 (equal or > 40 kg/m^2). Class 2 and class 3 are usually considered morbid obesity, these classes include also comorbidities secondary to obesity (*Engin 2017*; *Schwartz et al., 2017*). Obesity is a multifactorial disorder where genetic predisposition, environment, physiological, behavioural and epigenetic factors play a key role (*Heymsfield et al., 2017*). From a genetic point of view, recent evidence obtained from genome-wide association studies has revealed a high BMI inheritance rate, between 40 and 70%, and the involvement of over 140 genetic loci on BMI determination, mainly encoding components of leptin and melanocortin signalling pathways (*Fall et al., 2017*; *Bray et al., 2016*). Heterozygous mutations in the melanocortin-4 receptor encoding-gene are the best-known cause of monogenic obesity, and eleven rare monogenic forms of obesity due to a deficiency of leptin and melanocortin-4 receptors have been recognized (*Pigeyre et al., 2016*; *van der Klaauw et al., 2015*).

The accumulation of lipids, mainly in the form of triglycerides in the adipose tissue, the increase of liver volume and skeletal muscle, fat mass, β pancreatic cell mass, cardiac output and blood pressure, are the leading anatomical features in obesity (*Heymsfield et al., 2014*; *Hall et al., 2010*).

Obesity is also characterized by a low degree of chronic inflammation. Indeed, an increase in macrophages and other immune cells at the level of the adipose tissue was observed; these immune cells produce proinflammatory cytokines, which

contribute to the insulin resistance often found in obese patients (*Tchkonia et al., 2013*).

Free fatty acids (FFAs) produced by the hydrolysis of triglycerides in adipocytes are transferred to the bloodstream, in order to be used by cellular metabolism, and are elevated in plasma of obese patients. (*Tchkonia et al., 2013*). The excess of lipids is also internalized in the liposomes of the hepatocytes, where it can determine a pathological condition known as steatosis. Furthermore, the accumulation of lipids in different tissues can lead to lipotoxicity responsible for cellular dysfunction and apoptosis (*McCullough et al., 2004*). Lipid deposition in the tissues and increased plasma levels of free fatty acids and inflammatory cytokines are events contributing to the insulin resistance that characterizes many overweight and obese patients (*Tchkonia T et al., 2013*) (**Fig. 1**).

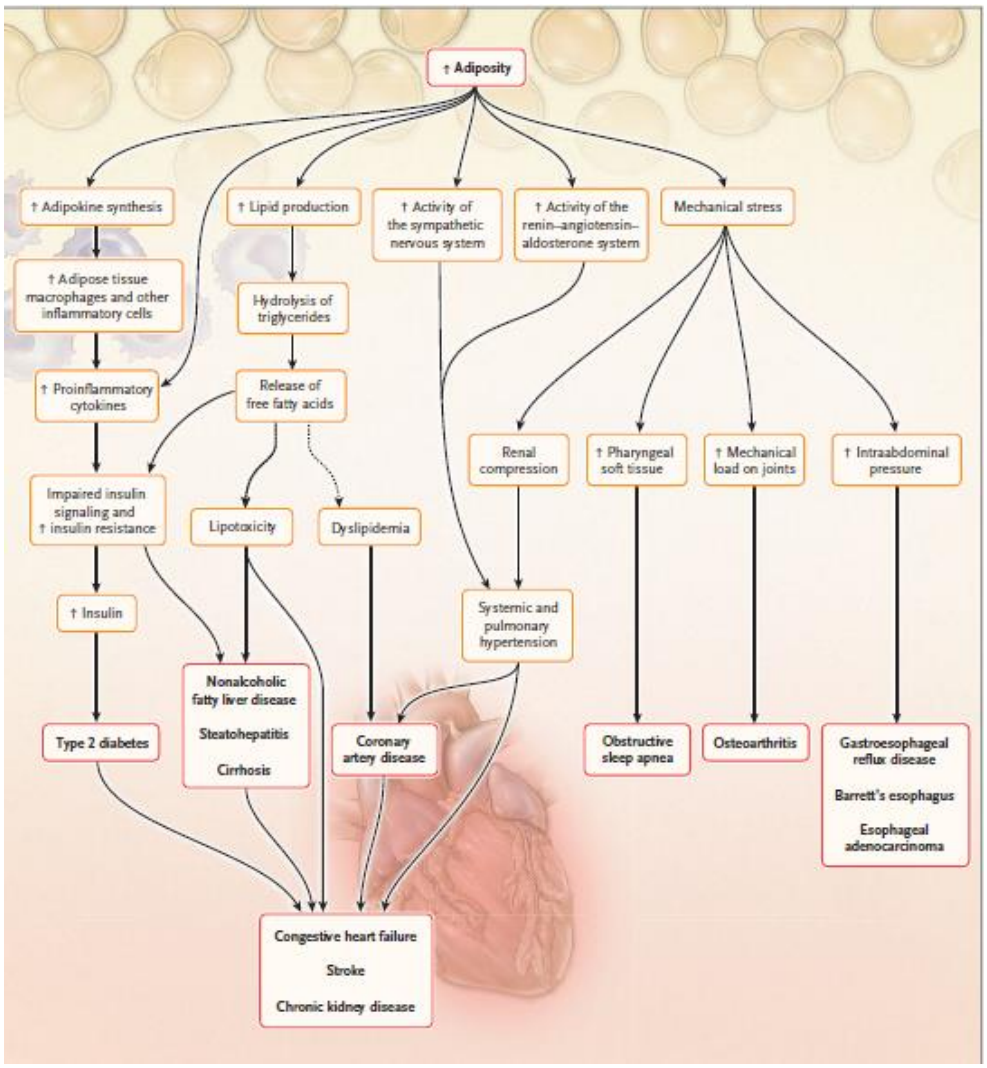


Figure 1. *Pathways activated by excessive adiposity leading to risk factors and chronic diseases. An indirect association is indicated by the dashed arrow (Heymsfield & Wadden, 2017).*

1.2 Metabolic syndrome (MetS): clinical and biochemical key features

Metabolic syndrome (MetS) is a common pathological condition defined as a cluster of different risk factors such as type 2 diabetes, hypertension, high triglycerides, low high-density lipoprotein cholesterol (HDL), central obesity and insulin resistance. In particular, central obesity and insulin resistance besides underlying MetS represent additional risk factors for the development of cardiovascular diseases (CVDs) (*Bonora 2006*). Visceral adiposity has been shown to be a trigger in MetS but, within the proposed activating mechanisms, insulin resistance, neurohormonal contribution and chronic inflammation appear to be the main actors involved in the onset and progression of the disease (*Matsuzawa et al., 2011*).

1.2.1 Hypertension and increased risk of CVDs

The metabolic syndrome is strongly associated with cardiovascular morbidity and mortality. The increased cardiovascular risk associated with MetS appears to be higher in women than in men and in individuals with pre-existing diabetes or cardiovascular diseases (*Bonora 2006*).

Obesity is also recognized as a contributing factor for the development of CVDs such as coronary artery disease, stroke, peripheral artery disease, cardiomyopathy, and congestive heart failure (*Poirier & Eckel, 2002*; *Chrostowska et al., 2013*). Several studies demonstrate a clear association between the risk of coronary heart disease (CHD) and an increased in BMI (*Rimm et al., 1995*; *Manson et al., 1995*).

1.2.2 Insulin resistance and impaired glucose tolerance

The increased FFAs is capable of exacerbating insulin resistance. In physiological conditions, insulin increases glucose uptake in muscle and liver, inhibiting lipolysis and hepatic gluconeogenesis. When insulin-resistance occurs in adipose tissue the inhibition of lipolysis induced by insulin is lost, consequently there is an increase in circulating FFAs, that may inhibit protein kinase activation in the muscle reducing glucose uptake. On the other hand, in the liver gluconeogenesis and lipogenesis are activated. The final effect is a prolonged hyperinsulinemic state (*Boden & Shulman, 2002*). Increased plasma levels of FFAs also cause lipotoxic effects in the pancreatic β-cells inducing decreased insulin secretion (*Tooke & Hannemann, 2000*). Insulin resistance represents a contributing factor to the development of hypertension due to the loss of the vasodilator effect of insulin while a FFAs-induced vasoconstriction persists (*Tripathy et al., 2003*). In addition, insulin resistance is able to induce the

release of pro-inflammatory cytokines from the adipose tissue that contribute to increase CVDs risk (*Juhan-Vague et al., 2003*).

1.2.3 Dyslipidemia

Atherogenic dyslipidemia is an important factor within the abnormalities characterizing MetS. Dyslipidemia associated to insulin resistance shows increased fasting and postprandial triglycerides, decreased HDL and increased low-density lipoproteins (LDL) (*Ruotolo & Howard, 2002*; *Expert Panel on Detection, Evaluation,and Treatment of High Blood Cholesterol in Adults JAMA 2001*).

2. Oxidative stress in metabolic and cardiovascular disorders

It is widely accepted that a crucial factor involved in the pathogenesis of several metabolic disorders is represented by oxidative stress. It is usually defined as a state of imbalance between oxidative agents and antioxidant defences, leading to increased production of free radicals and reactive oxygen species (ROS) (*Rani et al., 2016*). It is well known that excessive accumulation of fat in obese conditions and in the comorbidities characterizing MetS are associated with oxidative stress (*Manna & Jain, 2015*). The prolonged oxidative status leads to the damage of biomolecules (i.e. lipid peroxidation, protein oxidation and DNA damage), to cell and tissue dysfunctions that contribute to the development of metabolic disorders (*Rani et al., 2016*). Increased circulating levels of glucose and lipids are able to change the basal metabolism, and, being involved in several metabolic pathways, they may contribute to the rise of ROS production (*Marrocco et al., 2017*; *McMurray et al., 2016*).

A large number of studies underscores the existing correlation between oxidative stress and metabolic disorders such as obesity. An emerging hypothesis proposes oxidative stress as a link between fat accumulation-derived alterations and the onset of a cluster of health problems including adipokine secretion alteration, inflammation, and insulin resistance (**Fig. 2**) (*Savini et al., 2013*).

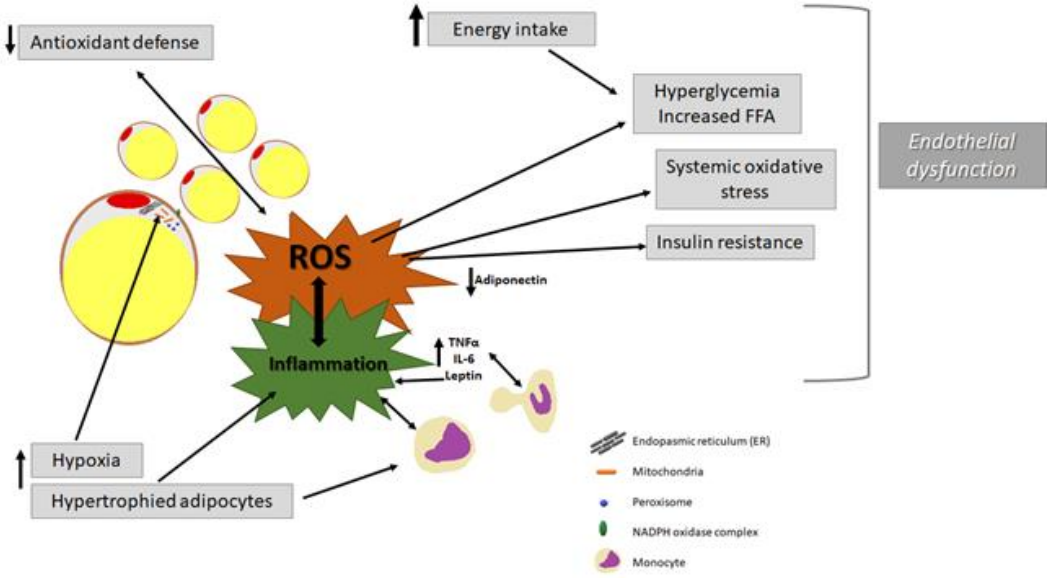


Figure 2. *Oxidative stress and inflammation during obesity. FFA, free fatty-acids; IL-6, interleukin 6; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; TNFa, tumor necrosis factor alpha (modified from Ruiz-Ojeda et al., 2018).*

CVDs are the most common cause of death worldwide, and a great number of these diseases have been shown the crucial role of excessive ROS production in their pathophysiology (*Senoner & Dichtl, 2019*). Although basal levels of ROS are essential for normal cellular functions, an increase in ROS concentrations may dramatically reduce nitric oxide (NO) availability with consequent vasoconstriction and arterial hypertension (*Finkel, 2011*; *Senoner & Dichtl, 2019*). One of the most important outcomes of free radical-dependent pathogenesis of CVDs is endothelial dysfunction. It is well known that the endothelium function is guaranteed by the production and release of various factors including vasodilator prostaglandins and NO. In addition, endothelial cells express the endothelial Nitric Oxide Synthase (eNOS), able to generate NO, which counteracts vascular damage and represents the main defense mechanism in the vascular system (*Forstermann & Munzel, 2006*; *Senoner & Dichtl W, 2019*).

The mechanisms behind endothelial dysfunction mainly concern a decreased production/availability of NO. The decreased NO availability may result from diverse factors, such as a reduced eNOS expression, the lack of substrates or cofactors for eNOS activity or the increase in NO degradation (*Antoniades et al., 2006*). Several cardiovascular risk factors, including hypercholesterolemia, arterial hypertension, and hyperglycaemia, are associated with an impaired endothelial function. Furthermore, endothelial dysfunction has also been linked to obesity and

the low degree of systemic inflammation underlying this metabolic disorder (*Favero et al., 2014*; *Hadi et al., 2005*). Literature data have demonstrated the crucial role of inflammation in the endothelial dysfunction that occurs in CVDs, obesity and type 2 diabetes mellitus. Studies deriving from both rodent and human models of endothelial dysfunction have shown increased levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1 β), interleukin-6 (IL-6) and interferon gamma (IFN -γ), secondary to the activation of the nuclear factor-kappa B (NF-κB) pathway. This important transcription factor regulates the expression of target genes involved in various processes such as cell adhesion, proliferation, inflammation, and redox balance (*Pierce et al., 2009*; *Wang et al., 2007*; *Lesniewski et al., 2011*; *Skoog et al., 2002*; *Dichtl et al., 1999*; *Dichtl et al., 2003*).

Oxidative stress acts as a main contributor not only to metabolic dysfunction, hypertension and heart failure but also to coronary heart disease and myocardial ischemia. In this regard, several studies showed the ROS involvement in myocardial acute ischaemia and on ischemia/reperfusion (I/R) injury (*Yellon & Hausenloy, 2007*; *Braunwald et al., 1985*). Accordingly, hypoxia and reoxygenation provoke an increase in free radical production in cardiac tissues and represent a major cause of reperfusion damage (*Yellon & Hausenloy, 2007*; *Braunwald et al., 1985*). The free radical production during this process is capable to induce both direct oxidative damage of cellular components and indirect injury through the activation of the inflammation pathway (*Griendling & FitzGerald, 2003*). Based on these evidences, it is conceivable to hypothesize that strategies aimed to counteract ROS formation may contribute to prevent and/or treat CVDs. Although the role of ROS in the onset and progression of vascular dysfunction and CVDs is unquestionable, and pre-clinical and small clinical studies indicated the ability of antioxidants to confer cardioprotection, antioxidant therapies show disappointing results in large-scale randomized controlled trials. Therefore, understanding the actions of the specific endogenous antioxidants (i.e., superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase) and exogenous antioxidants, as well as the functional crosstalk between them, by cellular and molecular approaches is crucial for understanding the limitations of antioxidant therapies in CVDs.

3. Structure and activity of mammalian selenoproteins

3.1 Selenoprotein identification and selenoproteome

Initially, several experimental approaches were used in order to identify the presence of selenium within the selenoproteins, in particular mass spectrometry

analysis and the detection of radioactive ^{75}Se, metabolically integrated in proteins in the form of the selenocysteine (Sec) residue, were performed (*Ballihaut et al., 2007*). The first identified selenoproteins were the mammalian glutathione peroxidase 1 (GPX1), bacterial glycine reductase and formate dehydrogenase (*Flohe et al., 1973*; *Turner & Stadtman, 1973*; *Andreesen et al., 1973*). Subsequently, thanks to a multidisciplinary approach many other selenoproteins were identified, and currently the full human selenoproteome, represented by 25 selenoprotein-encoding genes, is known (*Kryukov et al., 2003*) (**Fig. 3**).

Selenoprotein	Abbreviation	Sec location (protein size)	Function
Glutathione peroxidase 1	GPx1, GPX1	47 (201)	Cytosolic glutathione peroxidase
Glutathione peroxidase 2	GPx2, GPX2	40 (190)	Gastrointestinal glutathione peroxidase
Glutathione peroxidase 3	GPx3, GPX3	73 (226)	Plasma glutathione peroxidase
Glutathione peroxidase 4	GPx4, GPX4	73 (197)	Phospholipid hydroperoxide glutathione peroxidase
Glutathione peroxidase 6	GPx6, GPX6	73 (221)	Olfactory glutathione peroxidase
Iodothyronine deodinase 1	DI1, D1, DIO1	126 (249)	Thyroid hormone-activating iodothyronine deodinase
Iodothyronine deodinase 2	DI2, D2, DIO2	133, 266 (273)	Tissue-specific thyroid hormone-activating iodothyronine deodinase
Iodothyronine deodinase 3	DI3, D3, DIO3	144 (276)	Tissue-specific thyroid hormone-deactivating iodothyronine deodinase
Thioredoxin reductase 1	TR1, TrxR1, TXNRD1	498 (499)	Reduction of cytosolic thioredoxin
Thioredoxin/glutathione reductase	TGR, TR2, TrxR3, TXNRD3	655 (656)	Testis-specific thioredoxin reductase
Thioredoxin reductase 3	TR3, TrxR2, TXNRD2	522 (523)	Reduction of mitochondrial thioredoxin and glutaredoxin
Methionine-R-sulfoxide reductase	MsrB1, SelR, SelX, MSRB1	95 (116)	Reduction of oxidized methionine residues
Selenophosphate synthetase 2	SPS2, SEPHS2	60 (448)	Involved in synthesis of selenoproteins
Selenoprotein W	SelW, SEPW1	13 (87)	Unknown
Selenoprotein T	SelT	36 (182)	Unknown
Selenoprotein H	SelH	38 (116)	Unknown
Selenoprotein V	SelV	273 (346)	Unknown
Selenoprotein I	SelI, SEPI, EPT1	387 (397)	Unknown
15 kDa selenoprotein	Sep15	93 (162)	Putative role in quality control of protein folding in the ER
Selenoprotein M	SelM, SEPM	48 (145)	Unknown
Selenoprotein K	SelK	92 (94)	Putative role in ER-associated degradation
Selenoprotein S	SelS, SEPS1, VIMP	188 (189)	Putative role in ER-associated degradation
Selenoprotein O	SelO	667 (669)	Unknown
Selenoprotein N	SelN, SepN, SEPN1,	428 (556)	Putative role during muscle development
Selenoprotein P	SelP, SEPP1	59, 300, 318, 330, 345, 352, 367, 369, 376, 378 (381)	Se transport

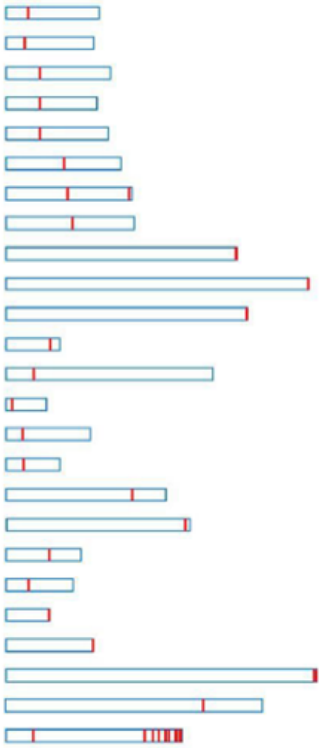
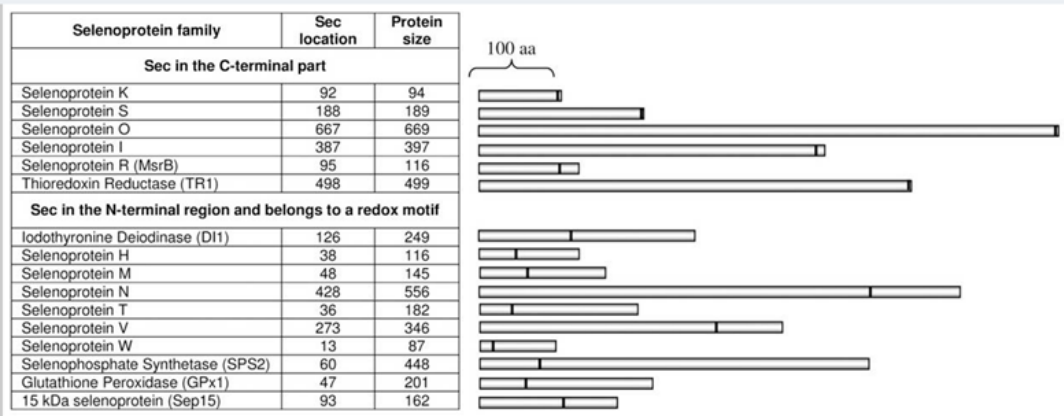


Figure 3. *Human selenoproteome. The right part indicates the relative length of selenoproteins and Sec location within the different selenoproteins (Kryukov et al., 2003).*

Considering that selenoprotein genes contain UGA codons, these proteins are often unannotated in sequence databases (*Kryukov et al., 2003*). An experimental approach aimed to identify selenoproteins was based on the two characteristic genomic features typical of these proteins, the in-frame UGA codon encoding Sec residue and the SECIS element (*Labunskyy et al., 2014*). By researching SECIS elements in completely sequenced genomes, and by analysing the genomes of species correlated from the evolution point of view, selenoprotein genes can be

identified through analysis of sequence (*Labunskyy et al., 2014 and references therein*). By another approach, selenoprotein genes were identified by searching the in-frame UGA codons through analysis of sequences near to UGA in sequenced genomes (*Labunskyy et al., 2014 and references therein*). Selenoproteins are present in bacteria, archaea and eukaryota. However, some organisms such as yeast and some plants losing Sec insertion machinery during evolution are unable to use Sec (*Lobanov et al., 2009*). Basing on the Sec location, mammalian selenoproteins are usually classified into two different groups (**Fig. 4**).



Selenoprotein family	Sec location	Protein size
Sec in the C-terminal part		
Selenoprotein K	92	94
Selenoprotein S	188	189
Selenoprotein O	667	669
Selenoprotein I	387	397
Selenoprotein R (MsrB)	95	116
Thioredoxin Reductase (TR1)	498	499
Sec in the N-terminal region and belongs to a redox motif		
Iodothyronine Deiodinase (DI1)	126	249
Selenoprotein H	38	116
Selenoprotein M	48	145
Selenoprotein N	428	556
Selenoprotein T	36	182
Selenoprotein V	273	346
Selenoprotein W	13	87
Selenophosphate Synthetase (SPS2)	60	448
Glutathione Peroxidase (GPx1)	47	201
15 kDa selenoprotein (Sep15)	93	162

Figure 4. *Human selenoproteins classified according to Sec localization. In the first group Sec is located very close to the C-terminus, often in the C-terminal penultimate position. In the second group Sec is located in the N-terminal or middle regions of selenoproteins, often in a redox motif within a thioredoxin fold (Lobanov et al., 2009).*

3.2 Antioxidant activity of selenoproteins

Selenoproteins represent a particular class of proteins that incorporate selenium, as an essential micronutrient, in the form of Sec. To date, all selenoproteins currently known exert important oxidoreductase activities by their redox active catalitic syte containing Sec and are able to catalyse redox reactions based on the oxidation of sulfhydryl groups and/or reduction of disulfides (*Steinbrenner & Sies, 2009*). Based on the location of Sec-residue, selenoproteins encompassing thioredoxin reductases (TXNRDs) and selenoprotein I (SELENOI), SELENOK, SELENOO, SELENOR, SELENOS, where Sec residue is located in the C-terminal region are classified in the first group, on the other hand selenoproteins including glutathione peroxidases, iodothyronine deiodinases, SELENOH, SELENOM, SELENON, SELENOT, SELENOV, SELENOW, SPS2, and Sep15, where Sec residue is in the N-terminal region and takes part in the redox-active thioredoxin (Trx)-like motif are ranked in the second group

(*Papp et al., 2007*). More than half selenoproteins contains a Trx-like fold, a key feature which in Trx includes two-layer α/β/α sandwich structure and a conserved motif made up of two Cys residues separated by two other amino acid residues (CXXC motif) (*Qi et al., 2005*). Alterations in the CXXC motif affects redox potential and the ability of the enzyme to interact with the substrates (*Chivers et al., 1996*; *Quan et al., 2007*). Selenoproteins such as SELENOM, SELENOT, SELENOP, SELENOW, and SELENOV include a CXXU motif that refers to the CXXC motif of the Trx active site and is crucial for their antioxidant activity. Despite the biological function of specific selenoproteins has been identified thanks to several approaches, other selenoproteins have unknown functions. Notably, the selenoproteins so far identified have redox functions and are involved in crucial biological processes, such as antioxidant defense, redox signaling and regulation, and intracellular homeostasis (*Zhang et al., 2020*).

3.2.1 Glutathione Peroxidase

Glutathione peroxidase (GPX) is an intracellular enzyme with antioxidant activity, mainly involved in the organism defence against oxidative stress. GPX catalyses the reduction of hydrogen peroxide, lipid hydroperoxides, and organic hydroperoxides to water or corresponding alcohols, by using GSH as cofactor (*Kieliszek & Blazejak, 2013*). Different evidence showed that GPX dysfunction is associated with the incidence of cancers (*Hu et al., 2005*; *Hu & Diamond, 2003*), muscle disorders (*El Haddad et al., 2012*), CVDs (*Rossmann 2011*; *Tanguy et al., 2011*), hepatopathies (*Carlson et al., 2016*), kidney failure (*Zachara et al., 2009*; *Taccone-Gallucci et al., 2010*), neuronal and neurodegenerative disorders (*Cardoso et al., 2017*; *Hambright et al., 2017*; *Chen et al., 2015*). The selenol in the Sec residue of GPX is oxidized by hydrogen peroxide or other oxidant agents, with consequent formation of selenenic acid (GPx-SeOH); the latter is reconverted to selenol by two-steps. Firstly, selenenyl sulfide (GPx-SeSG) is produced by the reaction between GPx-SeOH and GSH. Secondly, GSH reduces GPx-SeSG to selenol. The mammalian GPXs include eight isoforms, within of these GPX1 is ubiquitous in cytosol, GPX2 is gastrointestinal-specific, GPX3 is presents in plasma, while GPX4 (phospholipid hydroperoxide) and GPX6 (olfactory epithelium) can catalyse the reduction of H_2O_2 and lipid hydroperoxide with high efficiency (*Gromer et al., 2005*; *Neiers et al., 2007*). On the other hand, GPX6, GPX5, GPX7, and GPX8 are not selenoproteins and Sec residue is replaced by Cys residue (*Kryukov et al., 2003*).

3.2.2 Thioredoxin Reductase

TXNRD is an essential constituent of the TRX/TXNRD system, that is crucial for manteining the intracellular redox state (*Powis et al., 2000*; *Han et al., 2017*). To date, three isoforms of TXNRDs are found in mammals, in particular cytosolic TXNRD (TXNRD1), mitochondrial TXNRD (TXNRD2), and a testis-specific thioredoxin glutathione reductase (TXNRD3) (*Lu & Holmgren, 2009*). All the three enzymes contain the conserved part, Gly-Cys-Sec-Gly, (*Zhang et al., 2020 and reference therein*) where Sec exerts a critical role for TXNRD activity (*Zhong et al., 2000*). In mammalians, TXNRD1 and TXNRD2 have another conserved motif, localized in the N-terminal region known as Cys-Val-Asn-Val-Gly-Cys motif (*Lee et al., 2000*; *Cheng et al., 2009*). Thanks to the two conserved sites, mammalian TXNRDs have several properties and play crucial biological functions; in particular through direct TRX reduction, they can reduce glutaredoxin 2, protein disulfide isomerase, and many other disulfides in proteins and they can also reduce H_2O_2 (*Johansson et al., 2005*), selenite, lipid hydroperoxides, ascorbic acid, α-lipoic acid, cytosolic peptide granulysin, antibacterial NK-lysine, dehydroascorbate, and cytochrome C (*Zhang et al., 2020 and reference therein*). Similarly to other selenoproteins, the expression of TXNRDs is linked to the selenium concentration; indeed selenium deficiency reduced TXNRD synthesis and high concentrations of selenium can mediate Sec incorporation and increase TXNRD enzyme activity albeit not increasing protein synthesis. TXNRDs are enzymes ubiquitously expressed, able to regulate redox state. Several evidences demonstrated that the overactivation or dysfunction of TRX are associated with the incidence of diverse disorders, such as CVDs, neurological disease, type 2 diabetes and cancer (*Kondo et al., 2006*; *Arnér 2009*; *Holmgren & Lu, 2010*; *Gladyshev et al., 1999*). TRX1 besides acting as an antioxidant agent, plays an essential role in the regulation of cellular function by interacting with other molecules, such as agents involved in the inhibition of ventricular remodelling following a myocardial infarction (*Wande et al., 2020*).

3.2.3 Iodothyronine Deiodinases (DIOs)

The Iodothyronine Deiodinases (DIOs) family is characterized by selenoproteins containing a thioredoxin-fold, (*Labunskyy et al., 2014*). To date, three paralogous proteins in mammals (DIO1, DIO2 and DIO3) are known, which are all involved in the regulation of thyroid hormone activity. These proteins are located in different subcellular compartments and are expressed in several tissues; in particular DIO1 is strongly expressed in the liver, kidney, and thyroid, while DIO2 is expressed in the

brain, pituitary, thyroid, skeletal muscle, and brown adipose tissue. DIO3 was found in the cerebral cortex and skin, but is markedly expressed in the placenta and pregnant uterus (*Kuiper et al., 2005*). DIO1 and DIO2 catalyse the deiodination of the tetraiodothyronine (T4, inactive hormone form) into 3,3',5-triiodothyronine (T3, active hormone form); DIO3 converts T4 into reverse T3 and T3 into 3,3'-diidothyronine. DIO1 and DIO2 can also convert reverse T3 into 3,3'-diidothyronine (*Kuiper et al., 2005*).

3.2.4 Other selenoproteins

Despite the best characterized selenoproteins are TXNRDs, GPXs, and DIOs, other selenoproteins have an emerging role and most of them are implicated in several biological processes through their ability to regulate redox mechanisms.

Among them, SELENOS (also known as SEPS1, VIMP, and Tanis) is a transmembrane protein expressed in the liver, kidney, adipose tissue, skeletal muscle, pancreatic islets, and blood vessels (*Ye et al., 2016*). SELENOS is involved in the transport of unfolded or misfolded proteins from the endoplasmic reticulum (ER) to the cytoplasm, with consequent ubiquitin–proteasome-mediated degradation (*Smith et al., 2011*). It has been suggested that this selenoprotein plays an important role in inflammation processes, since it regulates pro-inflammatory cytokines release (*Fradejas et al., 2011*). Recent evidence demonstrated that genetic variants of SELENOS can associate with an increased risk of CVD. In this regard, specific SELENOS gene variants may be associated with an increase of the risk to onset of peripheral arterial disease (*Alanne et al., 2007*), on the other hand, genetic changes in SELENOS appear to prevent the risk to develop abdominal aortic aneurysm (*Strauss et al., 2018*). Other studies are mandatory to further elucidate the role of this selenoprotein in the pathogenesis of cardiovascular disorders.

SELENOK is another transmembrane selenoprotein exerting beneficial action against ROS-induced damage; in particular, it has been shown that is highly expressed in human heart and can reduce H_2O_2-dependent oxidative stress in cardiac cells (*Lu et al., 2006*). Other evidences have shown that this selenoprotein interacts with other protein and partecipates to the transport of misfolded proteins by regulating ER stress in particular cell types such as human liver cancer cells (HepG2), human embryonic kidney cells (HEK 293) and cervical cancer cells (HeLa) (*Rocca et al., 2019 and references therein*). In addition to the high expression of SELENOK in human heart, it is markedly expressed within the immune system cells, where it exerts essential action in cell migration and proliferation, and in the regulation of oxidative status (*Rocca et al., 2019 and references therein*).

SELENON is a transmembrane glycoprotein localized in the ER; it is highly expressed in most fetal tissues, but its level decreases in adult tissues. For instance, several evidences showed that SELENON is markedly expressed in fibroblasts and myoblasts, but its expression gradually decreases during the differentiation of these type of cells. SELENON exerts an essential role against oxidative stress and in Ca^{2+} homeostasis in human skeletal muscle cells (*Petit et al., 2003*; *Lescure et al., 1999*; *Arbogast et al., 2009*).

SELENOM is an ER-resident selenoprotein strongly expressed in the brain. This protein induces a protective action against oxidative stress by its redox active site thus contributing to intracellular calcium regulation. Pitts and collaborators have recently investigated the effects of SELENOM deficiency by using *knockout* (KO) mice. The authors showed that, despite SELENOM-KO mice did not exhibit deficits in motor coordination and cognitive function, they showed an increase in body weight, higher white adipose tissue deposition than wild-type, and diminished hypothalamic leptin sensitivity, suggesting that this selenoprotein could play an important role in the regulation of body weight and energy metabolism (*Pitts et al., 2013*).

3.3 Pathophysiological implications of selenoproteins

As mentioned above, due to the essential role exerted by specific selenoproteins in different biological processes, selective abnormalities related to this class of enzymes can associate to CVDs, neurodegenerative disorders, immune and endocrine disorders, liver disease, and cancer (*Papp et al., 2007*). Currently, many compounds containing selenium in organic form were tested as antioxidants, immunomodulators, antineoplastic and antihypertension agents. In this context, *Ebselen*, a GPX mimetic, was widely studied (*Muller et al., 1984*). For instance, it has been demonstrated that this drug, considered a peroxiredoxin-like agent, targets the thioredoxin system (*Zhao & Holmgren, 2002*) and exerts an effective action against H_2O_2 and smaller organic hydroperoxides inducing neuroprotective, antioxidant and anti-inflammatory actions in a rat model of transient cerebral artery occlusion (*Dawson et al., 1995*). These properties highlighted an application of ebselen in the treatment of patients with acute ischemic stroke (*Papp et al., 2007 and references therein*).

Several selenoproteins are known to play crucial roles in cerebrovascular health. Recent studies have reported that a reduction in serum SELENOP levels may occur in patients with stroke and may be linked to stroke (*Arteel et al, 1998*). This finding could be related to the crucial action exerted by this selenoprotein in brain. Indeed,

decreased levels of SELENOP, which is the main provider of selenium in the brain (*Schomburg L, et al., 2003*), could lead to a reduced protection against ROS (*Koyama et al., 2009*). Other studies reported reduced plasma levels of SELENOP, the main selenoprotein responsible for the transport of selenium from the liver to target tissues, in patients with a history of CVD that also present metabolic syndrome (*Rocca et al., 2019 and reference therein*). The data obtained so far provide potential clinical role of this selenoprotein as a biomarker for cardiometabolic pathologies and brain disorders.

Trx and TRXR1 are two enzymes strongly expressed in many types of cancer cells, in particular in non–small cell lung carcinoma (*Kakolyris et al., 2001*; *Soini et al., 2001*), malignant pleural mesothelioma (*Kahlos et al., 2001*), breast carcinoma (*Matsutani et al., 2001*; *Turunen et al., 2004*), colorectal cancer (*Raffel et al., 2003*), hepatocellular carcinoma (*Kawahara et al., 1996*) and other solid tumors (*Grogan et al., 2000*; *Hedley et al., 2004*); these enzymes are highly required for cancer cell proliferation and their overexpression may affect anticancer-drug resistance (*Papp et al., 2007*). Several studies also demonstrated that anticancer drugs such as cisplatin (*Arnér et al., 2001*) and doxorubicin exhibit inhibitor activities on TRXR activity. These findings provide important indication about the potential clinical application of these selenoenzymes as targets for cancer treatment (*Mau & Powis, 1992*).

4. Selenoprotein T (SELENOT)

Selenoprotein T (SELENOT) was the second selenoprotein identified by using a bioinformatic approach. SELENOT gene is localized on chromosome 3 in human and mouse while on chromosome 2 in rat; this gene includes 6 exons, where exon 2 contains the Sec-encoding TGA triplet, exon 5 contains the TAG stop codon and exons 5-6 contains the 3'-untranslated region including the stem-loop SECIS structure. Analyses performed by molecular and bioinformatic approaches showed that the SELENOT transcript contains 970 nucleotides encoding a pre-protein of 195 amino acids, which has a 19-amino acid signal peptide and a hydrophobic transmembrane domain (*Anouar et al., 2018*). SELENOT has a mass of 22.3 kDa and Sec residue is located at position 49 in the N-terminal region as part of the CVSU motif representing a redox site found in other selenoproteins that belong to thioredoxin-like family (*Grumolato et al., 2008*; *Moustafa et al., 2012*) (**Fig. 5**).

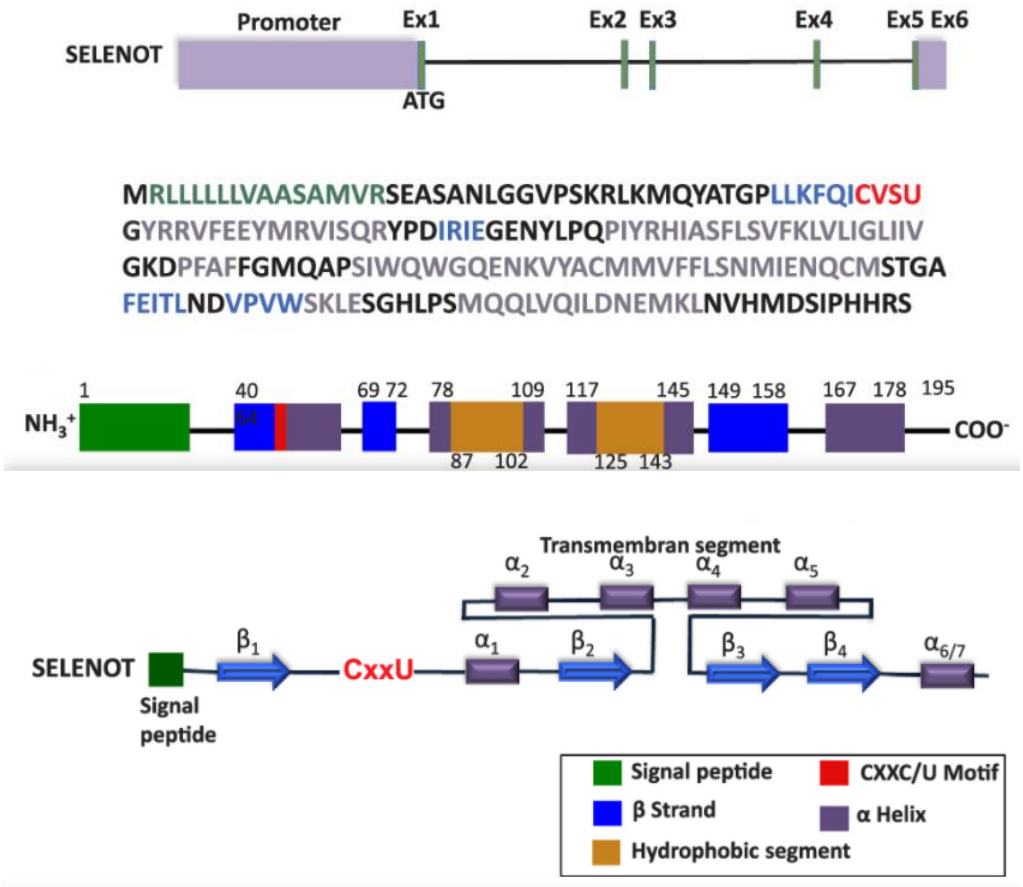


Figure 5. *Structure of SELENOT gene on chromosome 3 and correspondent primary and secondary protein sequence highlighting the signal peptide contained in preprotein, the presence of α-helix and β- sheets and the characteristic thioredoxin like-motif (Anouar et al., 2018).*

SELENOT is a plasma membrane protein localized into the Golgi apparatus and ER (*Moustafa & Antar, 2012*) and considered as a thioredoxin-like enzyme.

SELENOT has been identified as a novel target gene of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP). This evidence was suggested by the PACAP ability to induce a rapid increase in SELENOT gene expression during PC12 cell differentiation by a cAMP-dependent mechanism (*Grumolato et al., 2008*). It was found that SELENOT plays a crucial role in the regulation of Ca^{2+} homeostasis since its overexpression was able to induce an increase of the intracellular Ca^{2+} concentration in PC12 cells. Furthermore, by using site-directed mutagenesis of the Sec residue of SELENOT, it was demonstrated that PACAP stimulates the expression of SELENOT and its targeting to the ER to control Ca^{2+} release through a Sec-mediated redox mechanism. Accordingly, when Sec residue was replaced by Ala residue, the biological effect was abolished. Likewise, SELENOT *knockdown*

abrogated PACAP-stimulated release of Ca^{2+} from the ER (*Grumolato et al., 2008*). The mechanism of action by which SELENOT regulates intracellular Ca^{2+} and hormone secretion during PACAP-induced PC12 cell differentiation, is depicted in **Fig. 6.**

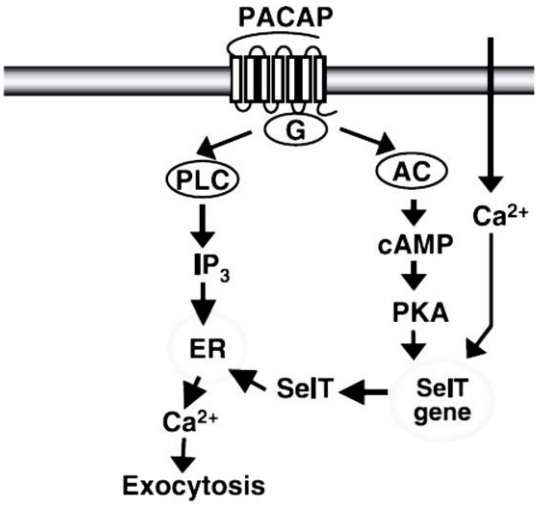


Figure 6. *Schematic model of SELENOT mechanism of action on intracellular Ca2 + and on hormonal secretion during PC12 cell differentiation induced by PACAP (Grumolato et al., 2008).*

4.1 Physiopathological implication of SELENOT in cardiometabolic and neurodegenerative diseases

4.1.1 Role of SELENOT against oxidative injury in nervous, endocrine, and metabolic tissues

SELENOT is relatively conserved in mammals and exerts an essential role in developmental processes. Accordingly, SELENOT knockout gene results in an early embryonic lethal phenotype in mice (embryonic day 8) (*Pothion et al., 2020 and references therein)* and is highly expressed during development; however, SELENOT expression levels decrease after birth and disappear in most adult tissues except in endocrine glands (pituitary, pancreas, testis, thyroid) (*Hamieh et al., 2017*; *Tanguy et al., 2011*; *Prevost et al., 2013*). Several experimental evidences showed that the expression of this selenoprotein is upregulated after exposure to noxious pathophysiological stimuli, in particular oxidative and ER stress, inflammation, heavy metal and neurotoxin exposure (*Pothion et al., 2020 and references and therein*). Notably, the genetic knockdown of this protein in corticotrope cells triggers an unfolded protein response (UPR) with consequent ER stress and ER dysfunction.

Additional data showed that SELENOT is highly expressed during adrenal ontogenesis but not in the adult adrenal gland; furthermore, Tanguy and colleagues (2011) observed that in adrenomedullary mature cells, PACAP was not able to stimulate SELENOT gene expression, suggesting that this selenoprotein may exert an important function during cell differentiation and tissue development where PACAP plays a major role (*Ghzili et al., 2008*). These data have been corroborated by *in situ* hybridization RT-qPCR, western blot and immunohistochemistry analysis, showing a high expression of SELENOT and its mRNA during embryogenesis, a progressive decrease during organ development while results absent in adult phase (*Grumolato et al., 2008*; *Tanguy et al., 2011*). SELENOT was undetectable in adult nervous cells except rostral migratory-stream astrocytes and Bergmann cells (*Tanguy et al., 2011*). On the other hand, SELENOT expression was maintained in several adult endocrine tissues such as pituitary, thyroid, or testis (*Tanguy et al., 2011*). According to these findings, as also observed in the case of other selenoproteins (i.e. SELENON, SELENOW, SELENOP and GPX), the biological significance of the greater SELENOT expression in fetal thyroid gland and testis, , may be related to the capability of the protein to control redox cell balance in order to counteract the significant oxidative burst that is physiologically generated during hormone biosynthesis (*Chung et al., 2009*; *Petit et al., 2003*; *Baek et al., 2005*; *Tanguy et al., 2011*). Noteworthy, it has also been reported that SELENOT expression is strongly stimulated in liver cells during the regenerative process that occurs after partial hepatectomy (*Tanguy et al., 2011*), suggesting the important role of this selenoprotein after tissue damage.

4.1.2 Involvement of SELENOT in pancreatic β-cell function and in the control of glucose metabolism

Growing evidences indicate that selenoproteins are involved in the regulation of glucose homeostasis and that an alteration of their synthesis is linked to diabetes progression (*Mueller et al., 2009*; *Labunskyy et al., 2011*). Prevost and colleagues (2013) observed that conditional pancreatic β-cell SELENOT *knockout* mice (SELENOT-insKO) exhibit an impairment of glucose tolerance, as shown by a significantly higher ratio of glucose to insulin ratio compared to *wild-type* mice. These findings indicate a deficit in insulin production/secretion in mutant mice (*Prevost et al., 2013*). By immunofluorescence staining the authors observed that SELENOT is strongly expressed in the adult mouse and human endocrine pancreas (**Fig. 7**), in particular in the islets of Langerhans, where the protein is mainly

expressed in insulin- and in somatostatin-producing cells. The same authors found that SELENOT-insKO exhibit, an alteration in the islet morphology, as revealed by their smaller size in comparison to wild-type mice (*Prevost et al., 2013*) demonstrating for the first time that SELENOT crucially contributes to blood glucose regulation during pancreatic β-cell stimulation and that it is essential for pancreatic islet function (*Prevost et al., 2013*).

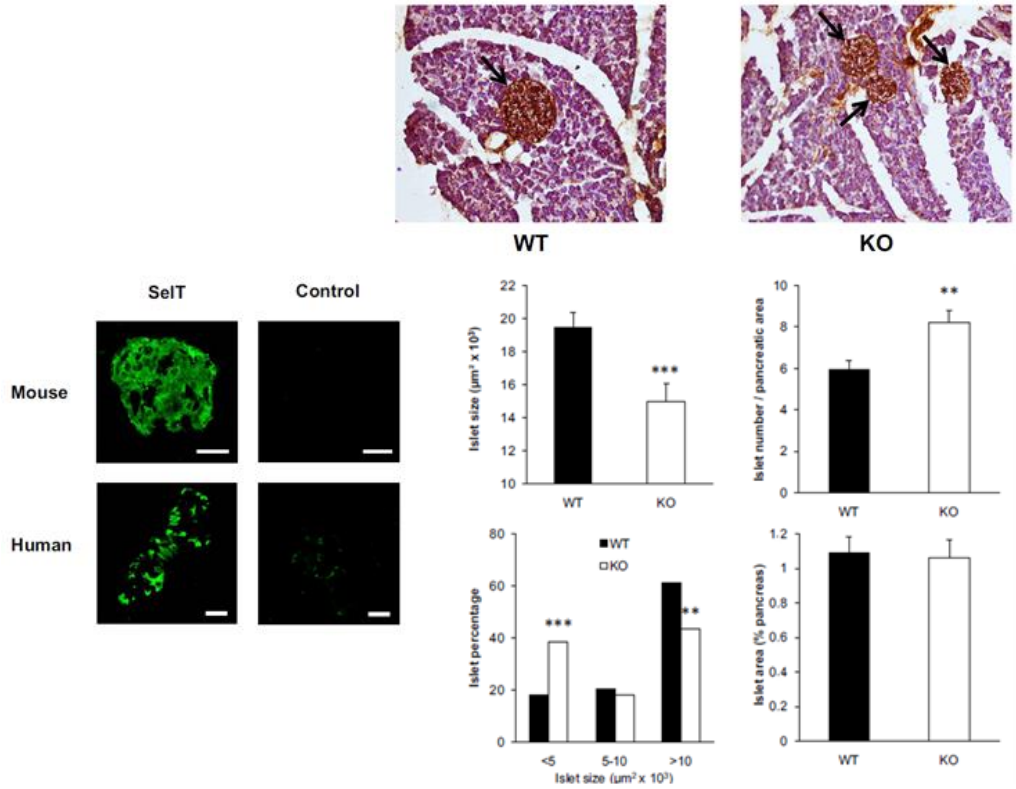


Figure 7. *SELENOT expression in mouse and human endocrine pancreas and morphological alterations in Langerhans islets in conditional SELENOT-insKO mice (Prevost et al., 2013).*

4.1.3 Role of SELENOT in oxidative stress-induced neurodegeneration in a model of Parkinson's Disease

As already mentioned, ROS are strongly involved in the pathogenesis of neurological disorders, including neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (PD) (*Wang et al., 2014*; *Blesa et al., 2015*). Since SELENOT is abundantly but transiently expressed during brain ontogenesis, and that its physiological function in the brain is not completely understood, Castex and colleagues (2016) and Boukhzar and collaborators (2016) evaluated the patho-physiological implications of SELENOT in the brain by using a conditional *knockout* mouse, in which SELENOT gene was specifically disrupted in nerve cells, and a mouse model of PD, respectively. In conditional SELENOT-KO mice in the brain, the

authors observed a significantly reduced volume of hippocampus, cerebellum, and cerebral cortex at postnatal day 7 (P7) (**Fig**. **8**), with a loss of immature neuronal cells, increased ROS levels and cell death. While, in the adult SELENOT deficiency led to cerebral dysfunction (*Castex et al., 2016*).

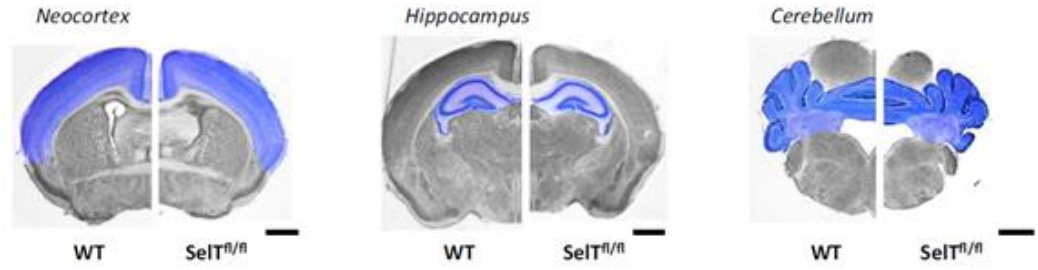


Figure 8. *Partial black and white" macrophotographs showing P7 brain sections stained with Nissl substance, within which the structures of interest appear in color (Castex et al., 2016).*

At the same time, Boukhzar and colleagues (2016) firstly demonstrated that SELENOT influences oxidative state and cell survival in SH-SY5Y cell model of dopaminergic neurons. Subsequently, they observed that specific neurotoxins (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP or rotenone), able to generate a PD-like phenotype in mice, stimulate SELENOT expression in the nigrostriatal pathway of wild-type mice and trigger a severe parkinsonian-like motor defect of conditional SELENOT-KO mice (*Boukhzar et al., 2016*). Furthermore, the authors revealed a remarkable increase of SELENOT in the caudate and putamen tissue of *post-mortem* PD patients (**Fig. 9**), providing insight about the crucial role played by this selenoprotein in the protection of dopaminergic neurons against oxidative stress and cell death (*Boukhzar et al., 2016*).

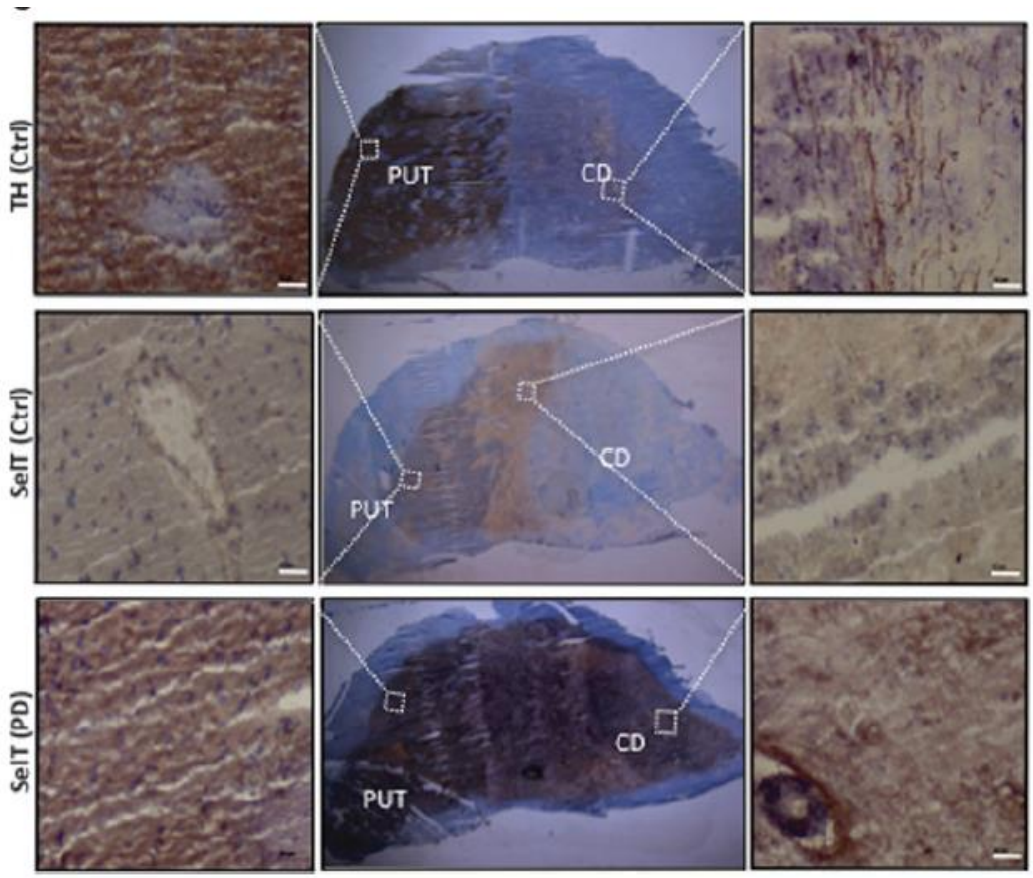


Figure 9. *Increased SELENOT expression in the caudate and putamen tissue of post-mortem PD patients (Boukhzar et al., 2016).*

4.1.4 Action of SELENOT in cardiac function after ischemia/reperfusion-induced injury

By analysing the SELENOT expression during the rat heart ontogenesis, Rocca and colleagues (2018) provided new insight into the biological role of SELENOT in the heart. They showed that the protein is very abundant in cardiomyocytes during embryonic ontogenesis of rat heart (E7), while its expression was reduced in newborn (post-natal day 14) and was undetectable in the adult heart, suggesting that SELENOT is required during early hyperplastic growth of cardiomyocytes. It is interesting to note that the ontogenetic expression pattern of SELENOT in heart is similar to that observed in the brain and other organs (as above reported), Furthermore, this study indicated that the adult rat heart exposed to ischemia/reperfusion damage is able to markedly re-express SELENOT (**Fig. 10**), thus arguing for a role of this selenoenzyme in adult heart exposed to a pathological stimulus. This finding is in line with the elevated expression of several selenoproteins under stressful conditions, which is

associated with protection against oxidative stress. Therefore, SELENOT expression during cardiac tissue ontogenesis and during ischemia is likely involved in cardiomyocyte protection and differentiation (*Rocca et al., 2018*).

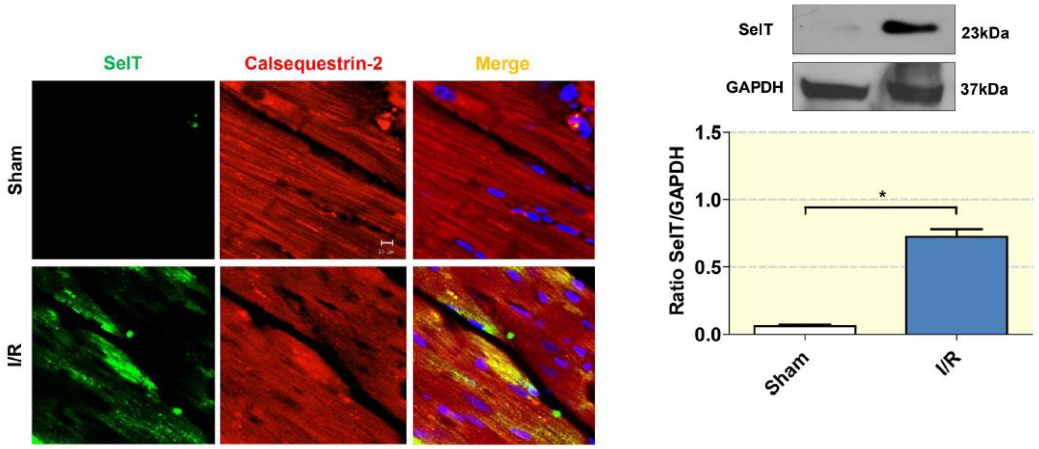


Figure 10. *SELENOT expression in Sham and I/R-treated rat hearts (Rocca et al., 2018).*

This observation prompted the authors to evaluate the cardioprotective mechanism of SELENOT in an *ex-vivo* induced I/R injury by employing a SELENOT-derived peptide, PSELT (FQICVSUGYR), encompassing the redox active site (CVSU) of the *full-length* protein. These results showed that PSELT was capable to significantly improve the post-ischemic systolic and diastolic function reducing infarct size as post-conditioning pharmacological agent. Notably, the observed effects were due to the catalytic site encompassing Sec, since an *inert* peptide lacking this residue did not exert any cardioprotective action. Mechanistically, this study showed that PSELT protected the heart form I/R insult by activating pro-survival signalling pathways, inhibiting apoptosis and decreasing oxidative and nitrosative myocardial stress (*Rocca et al., 2018*).

Aims of the doctoral project

Based on the knowledge described above, the present doctoral project was aimed to:

i) to investigate the protective effect, at systemic and cardiac level, of chronic administration of a SELENOT-derived peptide (SELENOT $_{43-52}$), PSELT, encompassing the redox motif (Cys-Val-Ser-Sec) of the *full-length* protein, on rat and mouse models of diet-induced obesity and metabolic syndrome;

ii) to elucidate the ability of PSELT in counteracting typical obesity- and metabolic syndrome-dependent complications, such as systemic and cardiac inflammation, glucose and lipid dysmetabolism, oxidative stress, and myocardial infarction susceptibility

Particularly:

• the first part of the work was designed to evaluate the beneficial effect of PSELT in an obesity rat model in:

- influencing "anthropometric" and metabolic parameters;
- inducing cardioprotective action studied at the end of treatment for evaluating the susceptibility to myocardial infarction in an *ex vivo*-induced I/R insult;
- reducing the release of specific marker of cardiac damage;
- protecting the cardiomyocytes from oxidative stress and palmitate-dependent damage *in vitro*;
- mitigating the myocardial, hepatic and aorta ultrastructural changes;
- evaluating the molecular mechanisms behind the protective effects of PSELT;

• the second part of the project was aimed to evaluate the beneficial action of PSELT in an obesity mouse model in:

- influencing "anthropometric" and metabolic parameters -ameliorating the anxiety-like phenotype and locomotor activity.

Materials and methods

1. Peptides and drugs

SELENOT-derived peptide $_{43\text{-}52}$ (PSELT) corresponding to the sequence FQICVSUGYR and an *inert* PSELT (corresponding to the PSELT sequence where Sec and Cys was replaced by Ser) were chemically synthesized with a method of solid phase on a Fmoc resin as previously described (*Chatenet et al., 2006*), using an Applied Biosystems model 433A peptide synthesizer (AB Sciex). Hydrogen peroxide solution (H_2O_2) and sodium palmitate has been purchased from Sigma Aldrich. All drug-containing solutions were freshly prepared before the experiments.

Experiments on rat model with diet-induced obesity

2. Animals

Male *Wistar* rats (Harlan Laboratories ENVIGO), weighing 250-300 g, were housed in a ventilated environment under standard conditions. The animals had access to food and water *ad libitum*. All experiments were conducted in accordance with the Italian law (DL. 26/14) and to the Guide for the Care and Use of Laboratory Animals, according to National Institutes of Health publication 85-23 (revised 1996). The project was approved by the Italian Ministry of Health, Rome, and by the Ethics Review Board of the University of Calabria.

2.1 Anthropometric parameters and body mass index measurement

Body weight, abdominal circumference and length were measured weekly in the different experimental groups, while food intake was recorded daily. Body mass index (BMI) was calculated at 12th week of treatment and expressed as ratio between body weight (g) and the square of the anal-nasal length (cm). After the sacrifice, abdominal, perirenal and epididymal and retroperitoneal fat of each rat was carefully weighed (*Casacchia et al., 2019*).

2.2 Experimental protocols

Male rats (8 weeks of age) were (*Casacchia et al., 2019*) with two different diets (purchased by ENVIGO s.r.l.) fed for 12 weeks and divided in four experimental groups:

Control (Normal Diet, **ND**): rats fed with standard diet (6,2% Kcal from fat, 44,2% carbohydrates, 18,6% protein) and i.p. treated with NaCl 0,9% in the last 28 days of diet;

Control + PSELT (or **ND + PSELT**)**:** rats fed with standard diet (6,2% Kcal from fat, 44,2% carbohydrates, 18,6% protein) and i.p. treated with PSELT 15 µg/kg/day in the last 28 days of diet;

Obese (High Fat Diet, **HFD**): rats fed with hypercaloric diet (60,3% Kcal from fat, 21,7% carbohydrates, 18,3% protein) and i.p. treated with NaCl 0,9% starting from the eighth week [time at which rats develop severe obesity and metabolic syndrome (*Nettore et al., 2019*; *Casacchia et al., 2019*)] for the rest of the treatment (last 28 days),

Obese + PSELT (or **HFD + PSELT**): rats fed with hypercaloric diet (60,3% Kcal from fat, 21,7% carbohydrates, 18,3% protein) and i.p. treated with PSELT 15 µg/kg/day in the last 28 days of diet.

2.3 Blood glucose and glucose tolerance test

For the assessment of blood glucose levels, glycemia was measured in fasted rats at the end of treatment. Furthermore, at the end of 12th week of diet, rats underwent an intraperitoneal glucose tolerance test (IPGTT) to evaluate glucose clearance. Rats were fasted for 12 hours with free access to water and then i.p. treated with glucose at 1.5 g/kg body weight. Blood glucose concentrations were evaluated using an IRIS EVOLUTION glucometer (IRIS HEALTH CARE). Each measurement was performed twice.

2.4 Biochemical Analysis

2.4.1 Plasma Metabolic Parameters

Blood samples were collected after sacrifice and centrifuged at 4 °C for 15 min at 4000 *x* g. Plasma was frozen at -80°C until use for biochemical analyses. Triglycerides, total cholesterol, LDL and HDL cholesterol were determined using a kit from PKL® POKLER ITALY.

2.5 Isolated heart perfusion according to Langendorff method

Rats were anesthetized with ethyl carbamate (2 g/kg rat) by intraperitoneal injection (i.p.). Hearts were rapidly harvested, transferred in ice-cold buffered Krebs-Henseleit solution (KHs) and immediately cannulated *via* aorta, that was connected to the Langendorff apparatus to start the retrograde perfusion (*Rocca et al., 2018*; *Rocca et al., 2019*). Perfusion was made at a constant flow-rate of 13 mL/min with oxygenated KHs, containing 113 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.1 mM mannitol, 11 mM glucose, 5 mM Na-

pyruvate, 1.8 mM $CaCl_2$, (Sigma Aldrich) (pH 7.4; 37 °C; 95% O_2 and 5% CO_2). The hearts were placed a controlled temperature of 37 °C. In order to prevent fluid accumulation, the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a pressure transducer (BLPR; WRI, Inc.), was inserted into the left ventricle through the mitral valve, to allow isovolumic contractions and to continuously record cardiac hemodynamic parameters. Another pressure transducer was localised above the aorta to measure coronary pressure (CP). The developed left ventricular pressure (dLVP, an index of contractile activity) and the left ventricular end-diastolic pressure (LVEDP, an index of contracture and cardiac damage) were measured to assess the cardiac contractility. The endurance of the preparations was stable until 190 min. The performance variables were measured every 10 min. Parameters were recorded by using the PowerLab data acquisition system (AD Instruments) as previously reported (*Pasqua et al., 2013*; *Rocca et al., 2018*; *Rocca et al., 2019*).

2.5.1 Ischemia/Reperfusion (I/R) protocol

Each heart was allowed to stabilize for 40 min with KH solution during which the baseline parameters were recorded. After stabilization period, the hearts were subjected to 30 minutes of global, no-flow ischemia followed by 120 minutes of reperfusion to induce I/R injury. Cardiac performance was evaluated in pre- and post-ischemic phases by analysing dLVP recovery, an index of contractile activity, and LVEDP, an index of contracture, defined as an increase in this parameter of 4 mmHg above the baseline level (*Rocca et al., 2018*).

2.6 Assessment of myocardial injury

2.6.1 Infarct size evaluation

To quantify the infarct area, at the end of reperfusion, the hearts were rapidly removed from the Langendorff apparatus. The left ventricles were dissected into 2-3 mm slices. After 20 min of incubation at 37°C in 0.1% nitro blue tetrazolium in phosphate buffer (59.8 mM NaH_2PO_4, 484.9 mM Na_2HPO_4, pH: 7.4), unstained necrotic tissues were accurately separated from stained viable tissues by an independent observer who was not aware of the nature of the intervention. The weights of the necrotic and non-necrotic tissues were then calculated, and the necrotic mass was expressed as a percentage of total left ventricular mass (% IS/LV) (*Pasqua et al., 2015*; *Rocca et al., 2018*; *Rocca et al., 2019*).

2.6.2 Assessment of brain natriuretic peptide (BNP)

The levels of BNP in myocardial tissues were analysed using Abcam Rat BNP 45 ELISA kit (ab108816) in accordance with the manufacturer's instructions.

2.7 Transmission electron microscopy

For transmission electron microscopy (TEM), cardiac and hepatic tissues and abdominal aorta of all experimental groups treated with or without PSELT were fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer pH 7.4 for 2 h at 4°C. After 2 hours, three washes were carried out for 10 min in phosphate buffer to remove any residual fixative, subconsequently post-fixation was performed in 1% osmium tetroxide (in 0.1 M phosphate buffer pH 7.4) for 2 hours at 4 °C in order to preserve the lipoprotein structures. Samples washed 3 times for 10 min with phosphate buffer, were underwent to gradual dehydration using increasing concentrations of acetone and then embedded in Araldite (Fluka, 10951). Ultrathin sections by using an RMC Power Tome series ultramicrotome and a Diatome diamond knife were prepared and collected on copper grids (EMS, G 300 Cu) to be examined with a Jeol JEM 1400 Plus electron microscope (Peabody, Massachusetts, USA) at 80 kV (Perrotta & Aquila, 2016).

Experiments on mice model with diet-induced obesity

3. Animals

Male C57BL/6 mice (Charles River Laboratories), weighing 22-24 g, were housed and kept in a ventilated room at 22 °C ± 2 °C temperature under a 12-hours light/12-hours dark cycle (light between 7:00 and 19:00). The animals had access to food and water *ad libitum*. All experiments were conducted in accordance with the European Committee Council Directives and approved by the Normandy Ethic Committee on Animal Experiments.

3.1 Experimental protocols

Male C57BL/6 (10 weeks of age) were fed for 16 weeks with two diets (purchased by Safe Complete Care Competence) and divided in four experimental groups:
Control (Normal Diet, **ND**): mice fed with standard diet (5,1% Kcal from fat, 52% carbohydrates, 21,4% protein);

Obese (High Fat Diet, **HFD**): mice fed with hypercaloric diet (36% Kcal from fat, 36,7% carbohydrates, 20% protein) and i.p. treated with NaCl 0,9% starting from the 1st week of diet;

Obese + PSELT 16 (or **HFD + PSELT 16**): mice fed with hypercaloric diet (36% Kcal from fat, 36,7% carbohydrates, 20% protein) and i.p. treated with PSELT 15 µg/kg three times a week starting from the 1st week of diet;

Obese + PSELT 8 (or **HFD + PSELT 8**): mice fed with hypercaloric diet (36% Kcal from fat, 36,7% carbohydrates, 20% protein) and i.p. treated with PSELT 15 µg/kg three times a week starting from the 9th week of diet.

3.2 Assessment of body weight, body composition and evaluation of food intake

Body weight starting from the first week were recorded every week until the end of the protocol (16th week) in the different experimental groups. Body composition was assessed on conscious animals during 16th week of treatment using MiniSpec LF110 (Bruker, Wissembourg, France) fast nuclear magnetic resonance method. Food intake measurements was recorded daily.

3.3 Blood glucose measurement

For the blood glucose determination, glycemia measurements were performed in mice fasted 6 hours (between 9:00 am to 3:00 pm) (*Jensen et al., 2013*) starting from 13th week of diet. Blood glucose concentrations were measured at various times using an AccuChek Performa glucometer (Roche Diagnostics). Each measurement was performed twice.

3.4 Behavioral analyses

3.4.1 Open field

The open field was used to assess anxiety-like behaviour and locomotor activity in mice exposed to the treatment indicated above. After 10 min of acclimatization, mice were individually placed in the center of the open field apparatus made up an infrared Plexiglass box with transparent walls and black floor (45 x 30 x 30 cm) (*Braga et al., 2013*). Mice have the intrinsic tendency to explore, however increased time spent in the marge zone is considered as indicative of anxiety state. Anxiety-like activities were recorded every 5 min for 15 min in a dim light room by computerized actimeter (Versamax, AccuScan Instruments, Inc., OH, USA). After completing the open field test to evaluate anxiety state, the infrared system was used to measure locomotor activity of mice from the different experimental groups in each compartment during consecutive periods of 6 cycles of 10 min in unlit and quiet room (*Seibenhener & Wooten, 2015*).

Effect of PSELT in an *in vitro* model of lipotoxicity and oxidative damage

4. Cell culture

H9c2 cell line (rat embryonic cardiomyocytes) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Thermo Fisher Scientific), and incubated at 37 °C in humidified atmosphere with 5% CO_2 (*Rocca et al., 2021*). When the cells reached a density of 80% in 100-mm plates, they were digested using 0.25% Trypsin-EDTA (1X) (Gibco) according to a 1:2 ratio following manufacturer's instructions (ATCC). For each experiment, cells were seeded and incubated for 48 hours at 37 °C, 95% O_2 and 5% CO_2.

4.1 Cell viability assay

The effects on H9c2 cell viability was assessed by 3-(4,5-dimethylthiazol-)2,5-diphenyl tetrazonium bromide (MTT) assay, as previously described (Rocca et al., 2021). Briefly, cells were seeded at a density of 5 × 10^3 cells/well in 96-well plate and firstly exposed to the following treatments: Control (vehicle) and Palmitate (PA) (100 µM, 200 µM, 300 µM, 400 µM and 500 µM) for 24 h. In addition, to evaluate the action of PSELT in palmitate-induced damage, we exposed cells to palmitate at first dose useful (100 µM) alone or in co-treatment with increasing concentration of PSELT (from 5 nM to 100 nM) for 24 h. The control was treated with the vehicle (BSA/ddH_2O).

Furthermore, in order to assess the effect of PSELT in hydrogen peroxide-induced oxidative damage, the cells seeded as above and they were pre-treated with PSELT (or *inert* PSELT) for 24 h and then exposed to hydrogen peroxide (H_2O_2) 200 µM for 3 h. The control was treated with the vehicle (0.9% NaCl).

At the end of the treatments, the cell culture medium was replaced with 100 µl of 2 mg/ml MTT solution (Sigma Aldrich) and cells were incubated for 4 h at 37 ◦C, 5% CO_2. After incubation, the solution was removed and the formazan crystals were solubilized by adding 100 µl of DMSO for 30 min. The absorbance was measured using a Multiskan EX Microplate Reader Lab (Thermo Fisher Scientific) at 570 nm. The means of absorbance values of six wells in each experimental group were expressed as the percentage of cell viability. The experiment was repeated three

times in independent way. Cell viability was reported as percentage of cells survival relative to control cells.

4.2 Oil Red O staining

Oil Red O staining provided the assessment of intracellular lipids accumulation in H9c2 cardiomyocytes. Briefly, H9c2 cells were seeded at a density of 5 x 10^4 cells/well and treated with PA 100 µM and 200 µM alone or together with PSELT. At the end of treatments, cells were processed for Oil Red O. H9c2 cells were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS three times, then followed by incubation with the Oil Red O working solution for 30 min at room temperature. After staining, cells were visualized with an Olympus BX41 microscope and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition.

4.3 Short interfering RNA (siRNA) transfection for SELENOT silencing

H9c2 cardiomyocytes were seeded at a density of 1 × 10^5 cells/well in 6-well plates 48 hours before transfection. For gene silencing, siRNA (100 nM) against SELENOT was transfected into H9c2 cells using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA). Scrambled siRNA used as control and siRNA for SELENOT were obtained from Santa Cruz Biotechnology. Six hours post-transfection, serum-free medium was replaced with growth medium and cells were incubated for 36 hours at 37 °C, 5% CO_2. The efficiency of SELENOT *knockdown* was assessed by western blot analysis. Afterwards, H9c2 cells were seeded at a density of 5 x 10^3 cells/well in 96-well plate and then transfected with SELENOT siRNA (si-SELENOT) or si-RNA negative control (si-NC) as above reported. After transfection, H9c2 cardiomyocytes were pre-treated with increasing concentrations of PSELT (from 5 to 1000 nM) for 24 h and then exposed to H_2O_2 (200 µM) for additional 3 h. At the end of treatments, H9c2 cell viability was evaluated by MTT assay as previously reported. The means of absorbance values of six wells in each experimental group were expressed as the percentage of cell viability that was reported as percentage of cells survival relative to si-RNA negative control cells. Experiments were repeated three independent times.

4.4 Western blot

At the end of the treatments, H9c2 cells were washed with DPBS (Gibco) and proteins were extracted by RIPA lysis buffer (Sigma-Aldrich) supplemented with

protease inhibitors (1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride, 200 mM sodium orthovanadate). Cells were centrifuged at 12000 x *g* for 15 min at 4 °C. Supernatant was collected for the assessment of protein concentration using Bradford reagent according to the manufacturer (Sigma-Aldrich). Equal amounts of proteins (50 µg) were separated on 12% SDS-PAGE gels (for SELENOT and β-Actin) exposed to electrophoresis and transferred to polyvinyl difluoride membranes (PVDF). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline with 0.5 % Tween 20 (TBST) for 45 min, then washed 3 times for 5 min with TBST and incubated overnight at 4 °C with different antibodies diluted in 5 % BSA in TBST. Anti-rabbit and anti-mouse peroxidase-linked secondary antibodies (Thermo fisher scientific) were 1:5000 and 1:2000 diluted in 5% non-fat dried milk in TBST, respectively. Immunodetection was performed by using the ECL substrates for high-sensitivity western blot detection (Thermo fisher scientific). Autoradiographs were obtained by exposing the membranes to X-ray films (Hyperfilm ECL, Amersham). Immunoblots were then digitalized the densitometric analyses of the bands were performed, then the background was subtracted. The analyses were conducted using ImageJ 1.6 (NIH).

5. Statistical analysis

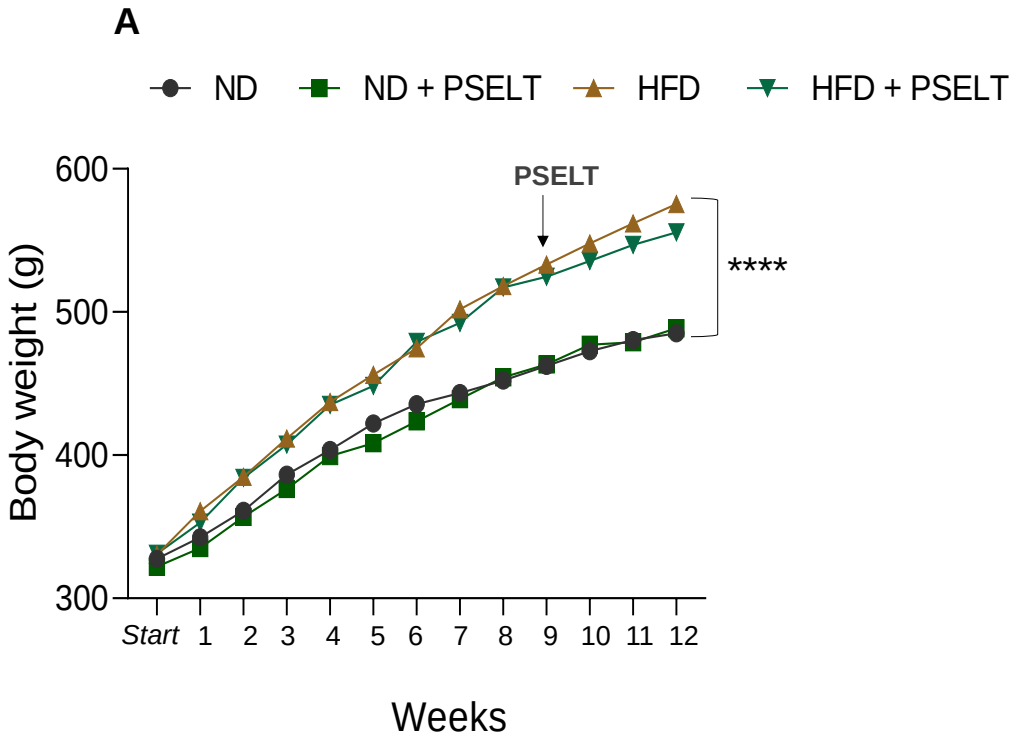
Data were expressed as mean ± SEM, Student's *t*-test, one-way ANOVA non-parametric Newman-Keuls Multiple Comparison Test or Bonferroni for post-ANOVA comparisons, Two-way ANOVA (non-parametric Bonferroni's multiple comparison test for post-ANOVA comparisons) were used when appropriate. Values of *p<0.05, **p =<0.01, ***p<0.001 were considered statistically significant. The statistical analysis was carried out using Graphpad Prism5 (GraphPad Software, California, USA).

Results

1. Effect of chronic administration of PSELT in a rat model of diet-induced obesity

1.1 Effect of PSELT on body weight, food intake and BMI

To evaluate the chronic effect of PSELT on weight gain induced by high fat diet, body weight was monitored starting from the first week of the dietary regimen. As depicted in **Fig. 11 A**, a significant increase in body weight was observed in HFD group compared to the control group during the 12th weeks of diet indicating that the model of diet-induced obesity was established. Data related to the experimental group fed with high fat diet and treated with PSELT (15 µg/kg/day) in the last 4 weeks showed a trend towards the reduction of body weight with respect to HFD alone (**Fig. 11 A**). No statistical differences were found in the HFD experimental groups treated with or without PSELT in term of food consumption, while food intake significantly decreased in HFD compared to ND group (**Fig. 11 B**). In addition, BMI significantly increased in the HFD group with respect to the control, and a slight trend towards a reduction, although not significant, was observed in obese rats treated with PSELT compared to HFD alone (**Fig. 11 C**).



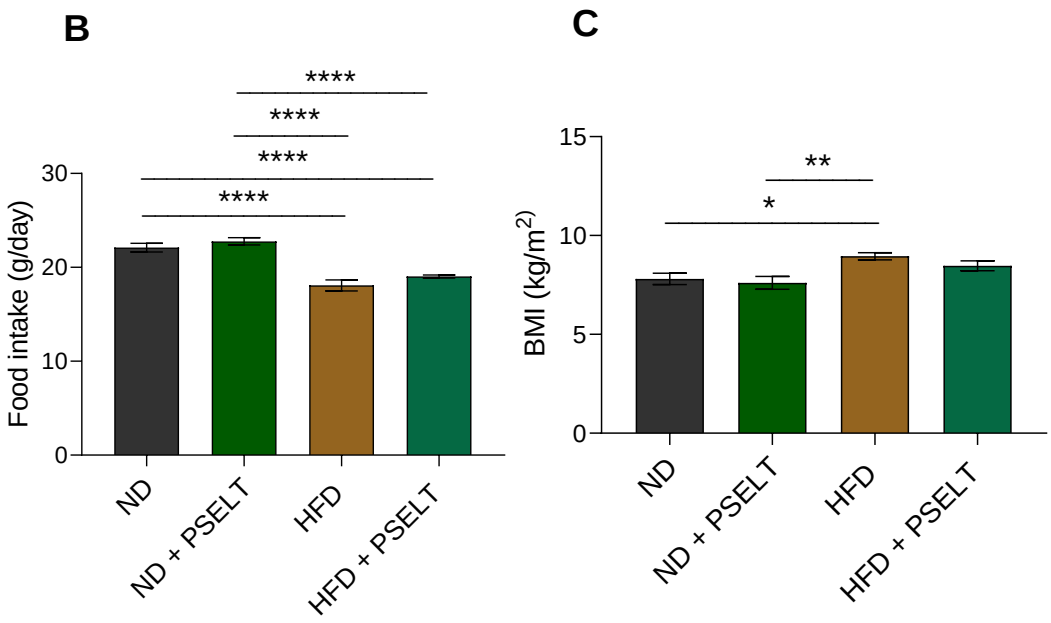


Figure 11. Effect of PSELT on body weight, food intake and BMI. A. *Body weight* ***B.*** *Food intake* ***C.*** *BMI in ND, ND + PSELT, HFD, HFD + PSELT. Data were expressed as the mean ± SEM. (n = 7 /group). Two-way ANOVA (analysis of variance) Bonferroni multiple comparison test was used for body weight analysis, One-way ANOVA and Newman-Keuls as post hoc were used for the evaluation of food intake and BMI in the different experimental group. Statistically significant differences: *=p < 0.05, **=p< 0.01, ****=p< 0.0001.*

1.2 Effects of PSELT on "anthropometric" parameters

Abdominal, perirenal, epididymal, retroperitoneal adipose tissues were collected and carefully weighed in order to assess fat accumulation and distribution in the different experimental groups. Data revealed that abdominal, perirenal, epididymal and retroperitoneal fat deposition were significantly elevated in HFD compared to ND while they significantly decreased in HFD + PSELT group compared to HFD alone (**Fig. 12 A, B, C, D**).

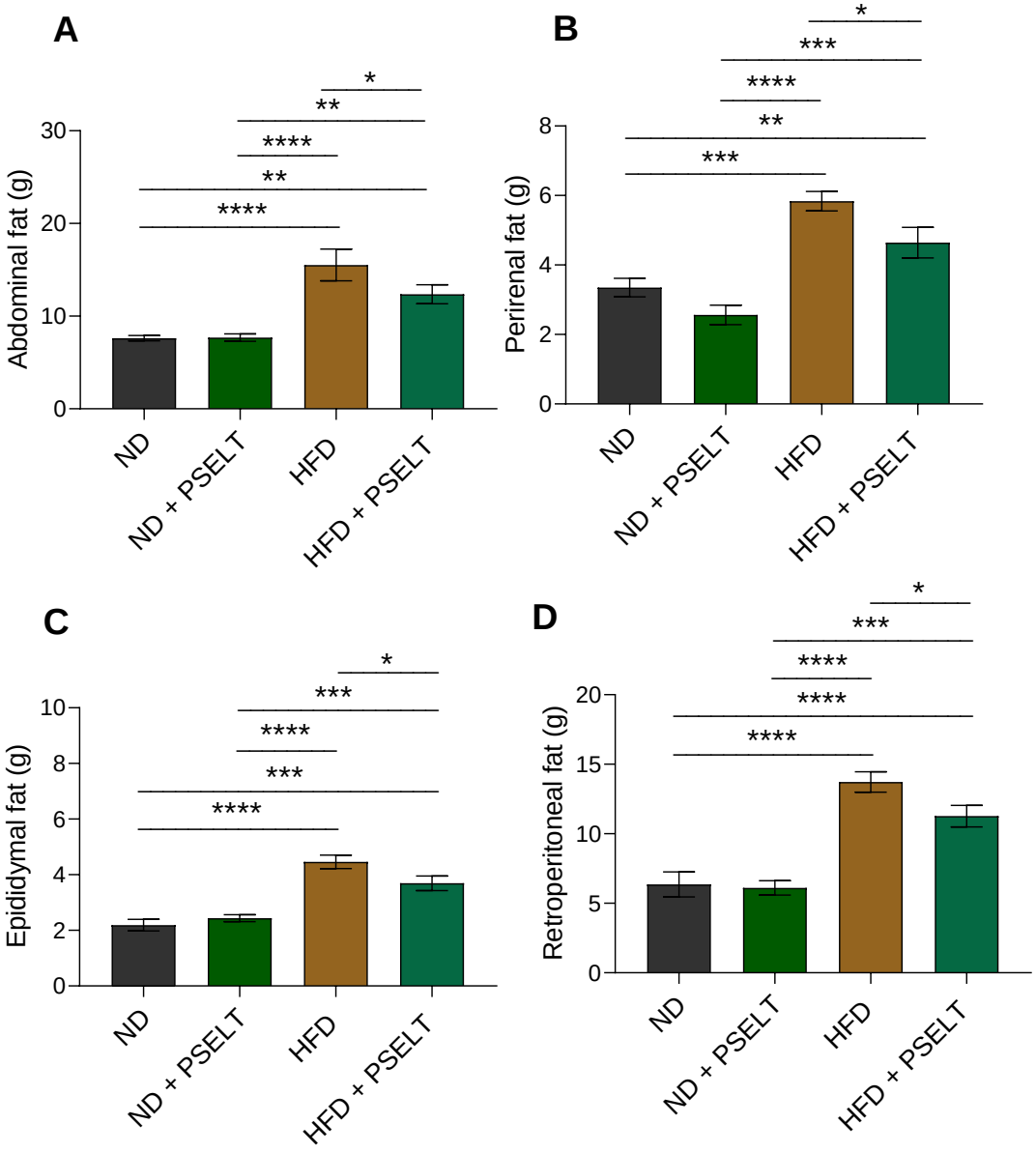
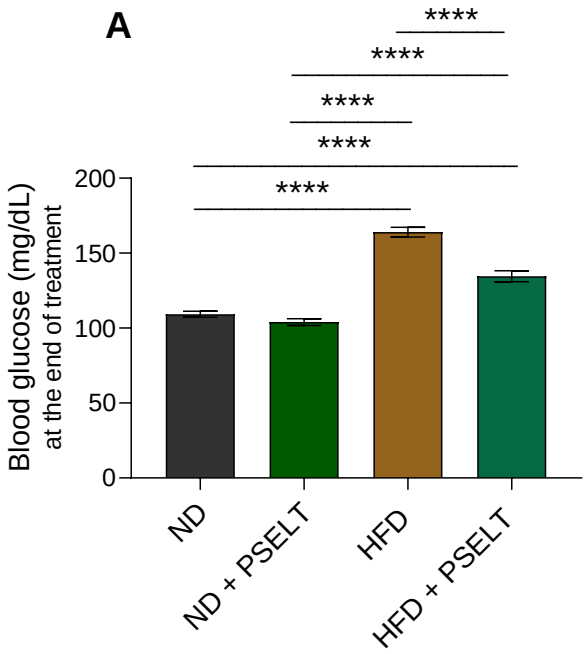


Figure 12. Action of PSELT on anthropometric variables. ***A.*** *Abdominal* ***B.*** *perirenal,* ***C.*** *epididymal and* ***D.*** *retroperitoneal fat in rats ND, ND + PSELT, HFD, HFD + PSELT, n = 7 for each group. Data were expressed as the mean ± SEM. One-way ANOVA, Newman-Keuls multiple comparison post hoc test. Statistically significant differences: *=p < 0.05, **=p< 0.01, ***=p< 0.001, ****=p< 0.0001.*

1.3 Action of PSELT on glucose metabolism in obese rats

Fasting blood glucose measurements were performed at the end of treatments (i.e. 12th week) in the different experimental group. Significantly higher levels of glycemia were observed in obese rats compared to the control rats, while a significant reduction in blood glucose was observed in HFD + PSELT in comparison

to HFD rats (**Fig. 13 A**). Furthermore, in order to evaluate insulin sensitivity, an IPGTT was carried out at the end of treatments in animals from each experimental group. Our data showed that HFD rats exhibit an impairment in glucose tolerance at 30, 60, 90 and 120 min after i.p. injection of 1.5 g/kg glucose compared to the ND rats; conversely, HFD rats treated with PSELT significantly improved glucose tolerance after 30 and 60 min of glucose i.p. administration (**Fig. 13 B**). The effect of PSELT in rats fed with ND was similar to ND alone group (**Fig. 13 A, B**).



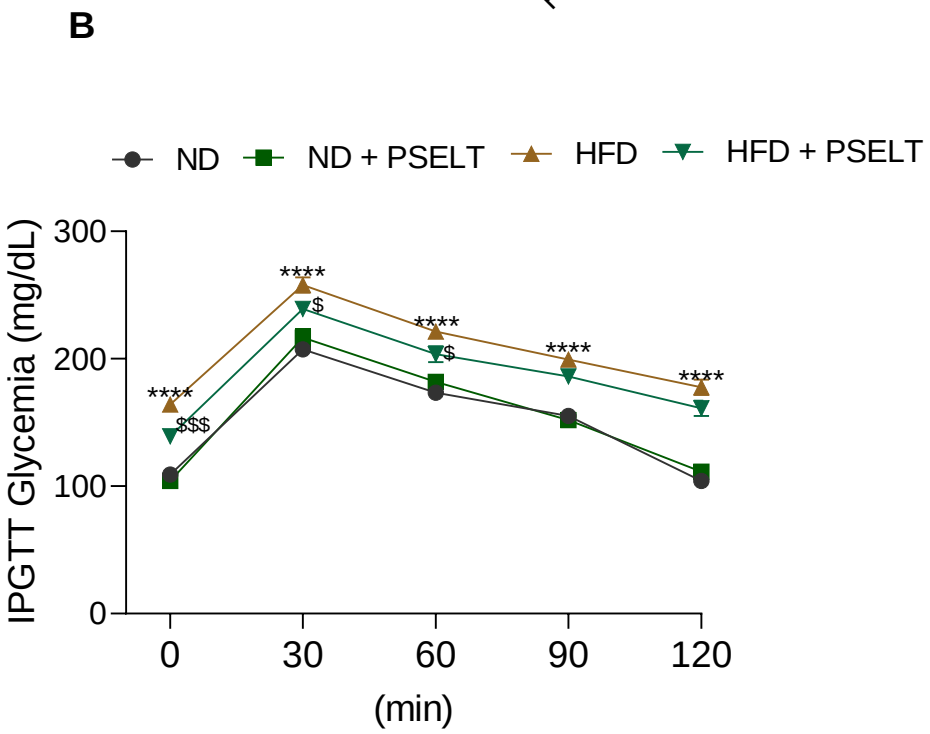
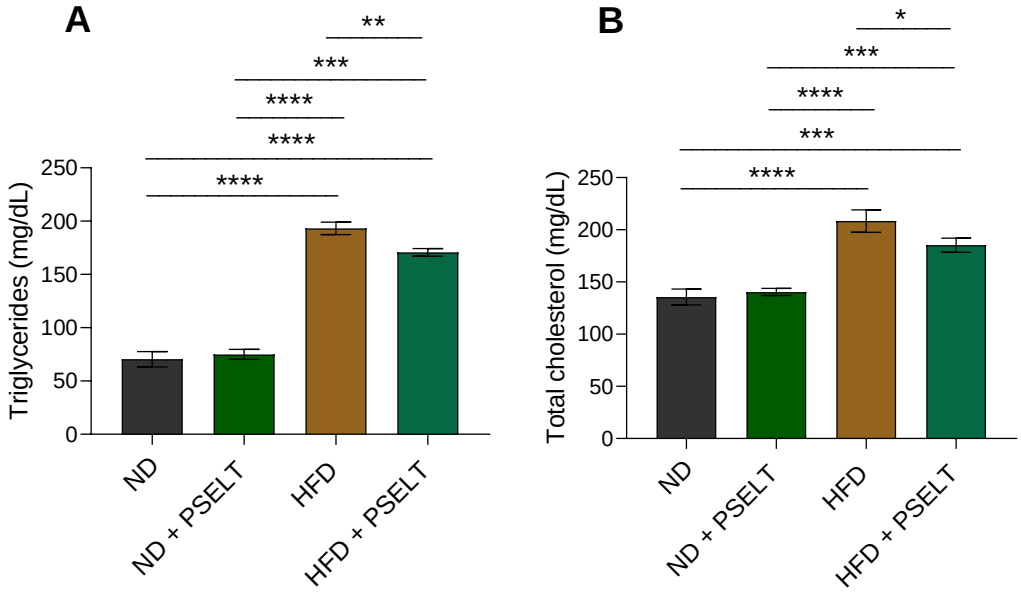


Figure 13. Role of PSELT on glucose metabolism. ***A.*** *Glycemia at the end of treatment in rats ND, ND + PSELT, HFD, HFD + PSELT, n = 5 for each group. Data were expressed as the mean ± SEM. One-way ANOVA, Newman-Keuls multiple comparison was used as post hoc test. Statistically significant differences: ****p< 0.0001.* ***B.*** *Intraperitoneal glucose tolerance test (IPGTT) in ND, ND + PSELT, HFD, HFD + PSELT n = 5 for each experimental group. Data were expressed as the mean ± SEM. Two-way ANOVA Bonferroni post hoc test was used. Statistically significant differences: ****p< 0.0001 vs ND, $p< 0.05, $$$p< 0.001 vs HFD.*

1.4 Effects of PSELT on lipid metabolism

The action of PSELT on dyslipidaemia induced by high fat diet was also investigated. To this aim, plasma triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol were analysed at the end of treatments. All these parameters were significantly altered in HFD-treated rats compared to ND, while the treatment with PSELT in HFD + PSELT group improved both lipid and lipoprotein profile (**Fig. 14**). In particular, triglycerides, total cholesterol and LDL cholesterol were significant augmented in HFD group with respect to ND, while HDL cholesterol showed a reverse trend (**Fig. 14 A, B, C, D**). On the contrary, the treatment with PSELT in rats fed with HFD was able to significantly reduce triglycerides, total cholesterol and LDL cholesterol plasma levels (**Fig. 14 A, B, C**) by also inducing a trend towards the increase HDL cholesterol compared to HFD alone group (**Fig. 14 D**). PSELT treatment in normal rats (ND + PSELT group) did not exert any significant action on lipid profile compared to ND alone **(Fig. 14**).



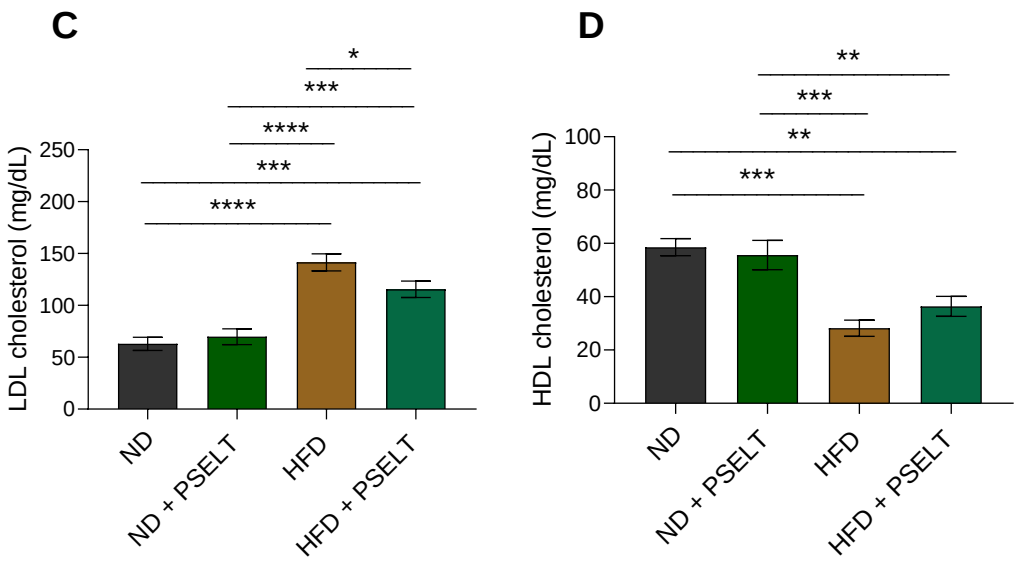


Figure 14. Effects of PSELT on lipid profile. ***A.*** *Plasma triglycerides* ***B.*** *Total cholesterol* ***C.*** *LDL cholesterol* ***D.*** *HDL cholesterol in ND, ND + PSELT, HFD, HFD + PSELT n = 7 for each experimental group. Data were expressed as the mean ± SEM. One-way ANOVA, Newman-Keuls multiple comparison was used. Statistically significant differences: *=p< 0.05, **=p< 0.01, ***=p< 0.001, ****=p< 0.0001.*

1.5 Pre-conditioning effects of chronic administration of PSELT on post-ischemic systolic and diastolic recovery in both normal and obese rats

The ability of PSELT to exert cardioprotective effects in a rat model of diet-induced obesity was investigated by studying the susceptibility to *ex-vivo*-induced myocardial I/R injury according to Langendorff technique. The basal cardiac parameters of all experimental groups after 40 min of stabilization are indicated in **Table 1** showing that the hearts from the ND + PSELT group had significant higher values of dLVP compared to ND alone, and that HFD group exhibited lower dLVP compared with controls (ND). Conversely, the hearts from HFD + PSELT significantly improved dLVP with respect to HFD alone group. The time course analysis and the graphs showing the values at the end of reperfusion depicted in **Figure 15 A, B** showed that in the ND and HFD groups, the dLVP at the end of reperfusion was significantly lower than before ischemia. In contrast, in the ND + PSELT and HFD + PSELT groups, dLVP significantly increased in the recovery phase compared to ND and HFD alone groups, respectively. Furthermore, in both the ND and HFD groups, the LVEDP significantly increased during reperfusion, whereas in the ND + PSELT

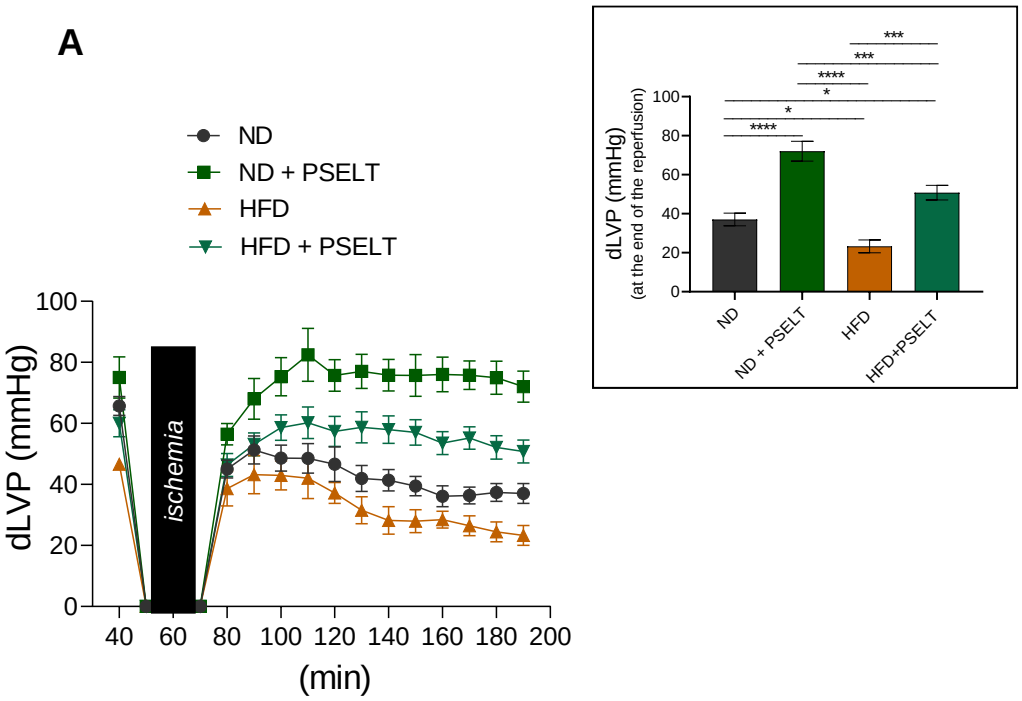
and HFD + PSELT groups, this parameter did not significantly change compared to their control counterparts. (**Fig. 15 B**).

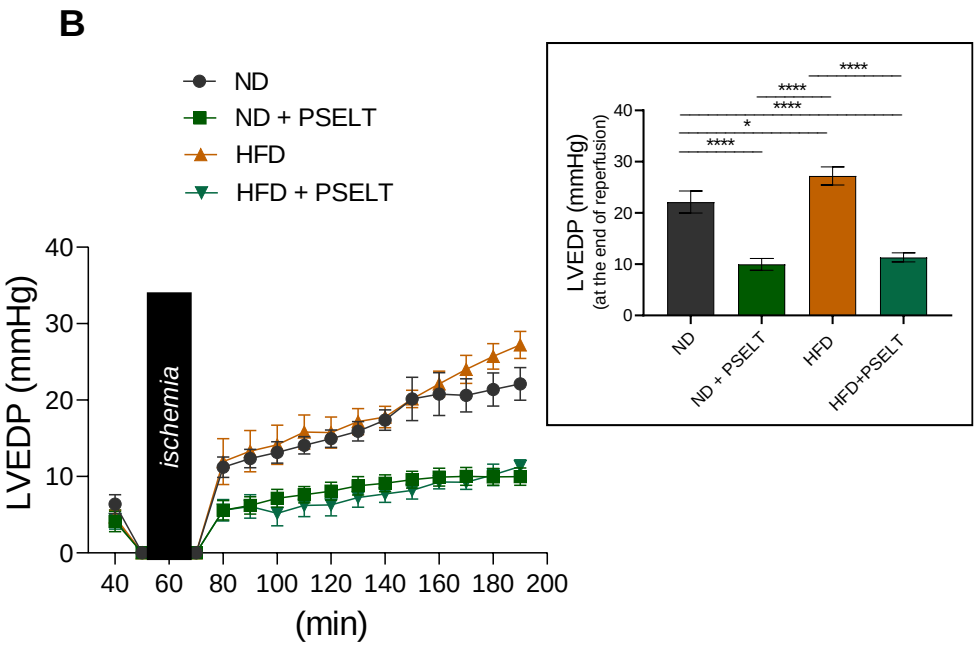
At the end of the reperfusion, the effect of PSELT on ischemia-induced infarct size (IS) [expressed as percent of the LV mass] was also examined. Control and obese groups showed significant increase of the infarct area, while in both the experimental groups treated with PSELT, the infarct size significantly decreased (**Fig. 15 C**).

TABLE 1. *Basal cardiac parameters after stabilization*

Experimental groups	dLVP (mmHg)	LVEDP (mmHg)
ND	65.7 ± 3.1	6.4 ± 1.3
ND + PSELT	75 ± 6.8	4.1 ± 1.4
HFD	$46.7^{*\#}$ ± 1.5	4.7 ± 0.9
HFD + PSELT	$60.1^{\dagger}$ ± 4.5	4.4 ± 1.3

dLVP, developed Left Ventricular Pressure; **LVEDP**, Left Ventricular End-Diastolic Pressure. $^{*}p< 0.05$, ND *vs.* HFD; $^{\#}p< 0.001$ ND + PSELT *vs.* HFD, $^{\dagger}p< 0.05$ HFD *vs* HFD + PSELT. (one-way ANOVA and Newman-Keuls multiple comparison test).


A
ND
ND + PSELT
HFD
HFD + PSELT
dLVP (mmHg)
ischemia
(min)
dLVP (mmHg)
(at the end of the reperfusion)
ND
ND + PSELT
HFD
HFD+PSELT


B
ND
ND + PSELT
HFD
HFD + PSELT
LVEDP (mmHg)
ischemia
(min)
LVEDP (mmHg)
(at the end of reperfusion)
ND
ND + PSELT
HFD
HFD+PSELT

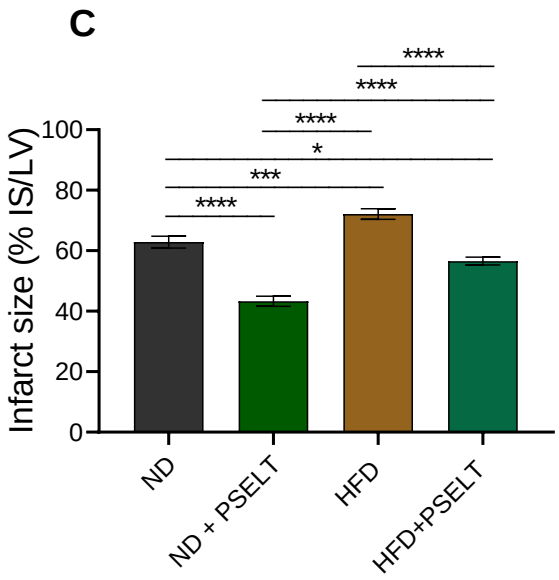


Figure 15. The effect of PSELT on I/R injury and infarct size in rat model with diet-induced obesity. A. *dLVP* ***B.*** *LVEDP variations. Black boxes indicate ischemic administration. Two-way ANOVA (analysis of variance) Bonferroni multiple comparison test was used. dLVP = 19.82 % of total variation between groups (P <0.0001); LVEDP =21.17 % of total variation between groups (P < 0.0001). Inset graph shows dLVP and LVEDP at the end of reperfusion. Data are expressed as changes of dLVP and LVEDP values (millimeters of mercury, mmHg) from stabilization to the end of the 120 min of reperfusion with respect to the baseline values for ND, ND + PSELT, HFD and HFD + PSELT groups (n = 7 hearts/group). *p < 0.05, ***p < 0.001, ****p < 0.0001 (One-way ANOVA and Newman-Keuls multiple comparison test).* ***C.*** *IS (n = 7 hearts/group). The amount of necrotic tissue measured after 30 min global ischemia and 120 min reperfusion is expressed as a percentage of the LV mass (% IS/LV) *p < 0.05, ***p < 0.001, ****p < 0.0001 by One-way ANOVA and Newman-Keuls multiple comparison test.*

1.6 Effect of PSELT on the ultrastructural cardiac changes induced by high fat diet

The influence of PSELT on cardiac ultrastructure changes in control and obese rats was assessed by transmission electron microscopy (TEM). Ultrastructural analysis revealed that in the control experimental groups treated with or without PSELT, the myocardial architecture was unaffected (**Fig. 16**). In both standard experimental groups (ND and ND + PSELT), nuclei were observed at the level of the medial zone of the myocardial cells, as well as a cytoplasmic area enriched in mitochondria aggregated around the nucleus or below the sarcolemma and/or located between the myofibrils. On the contrary, strong ultrastructural changes were observed in myocardial cells of animals fed with HFD. Additionally, cytoplasmic accumulations of lipid droplets and lipofuscin (a marker of oxidative stress) granules, as well as focal myofibrillar degeneration were found in the hearts of HFD rats compared to

ND rats. Conversely, PSELT was able to suppress the cytoplasmic accumulation of lipid and lipofuscin granules secondary to HFD regimes, restoring the structural integrity of the cardiomyocytes (**Fig. 16**).

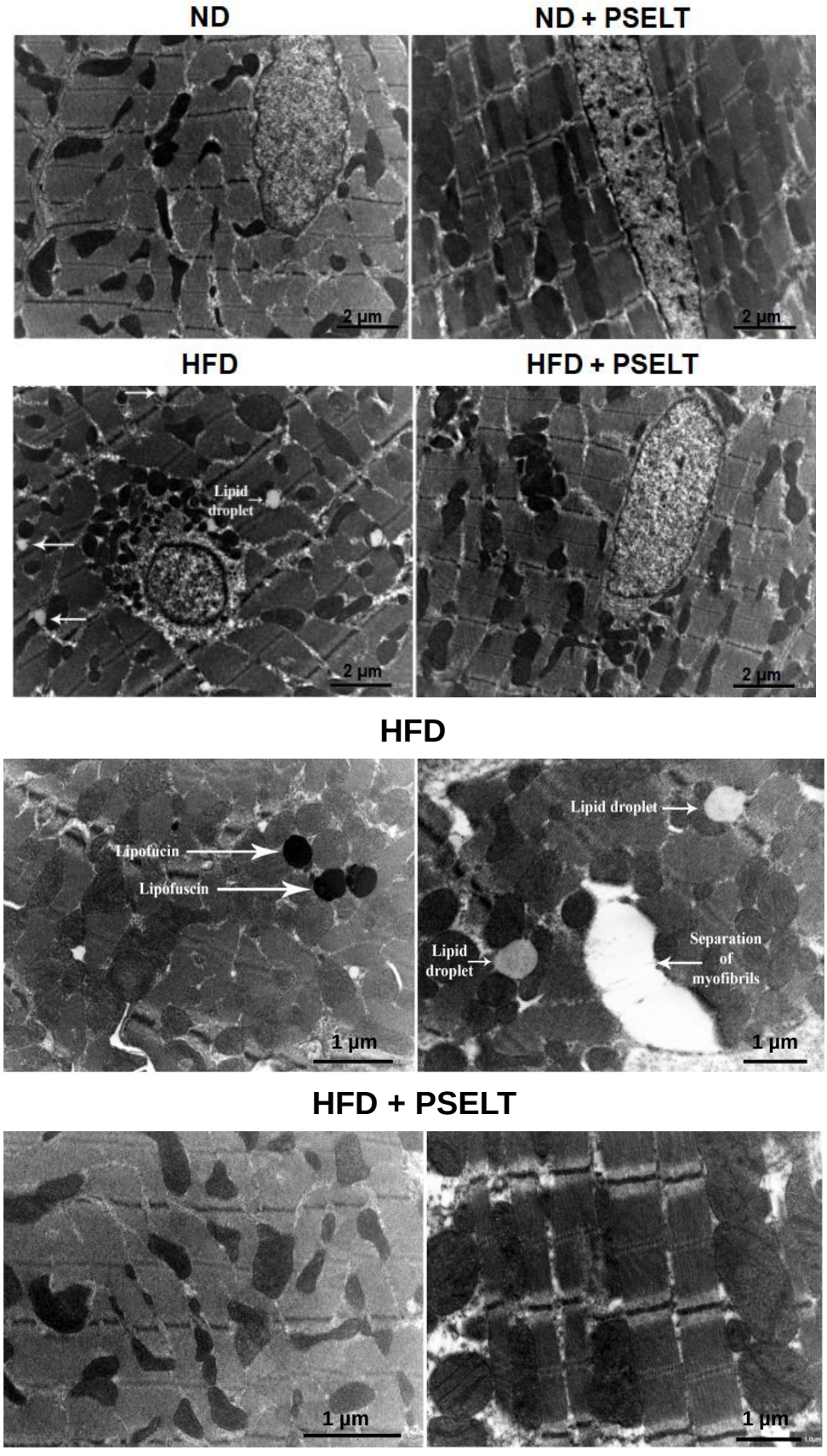


Figure 16. Action of PSELT on cardiac structure in control and obese conditions. *Ultrastructure representations obtained by transmission electron microscopy (TEM) of cardiac sections in ND, ND + PSELT, HFD, HFD + PSELT experimental groups. (Scale bar: 2 µm and scale bar: 1 µm).*

1.7 Action of PSELT on hepatic changes induced by high fat diet

Hepatic ultrastructure was also analysed to establish whether PSELT could improve HFD-dependent liver alterations. Our data obtained by TEM analyses showed that liver structure was preserved in ND and ND + PSELT experimental groups, while liver from HFD rats displayed steatosis due to excessive fat accumulation. However, both hepatic steatosis and microarchitecture were ameliorated in HFD + PSELT group compared to HFD (**Fig. 17**).

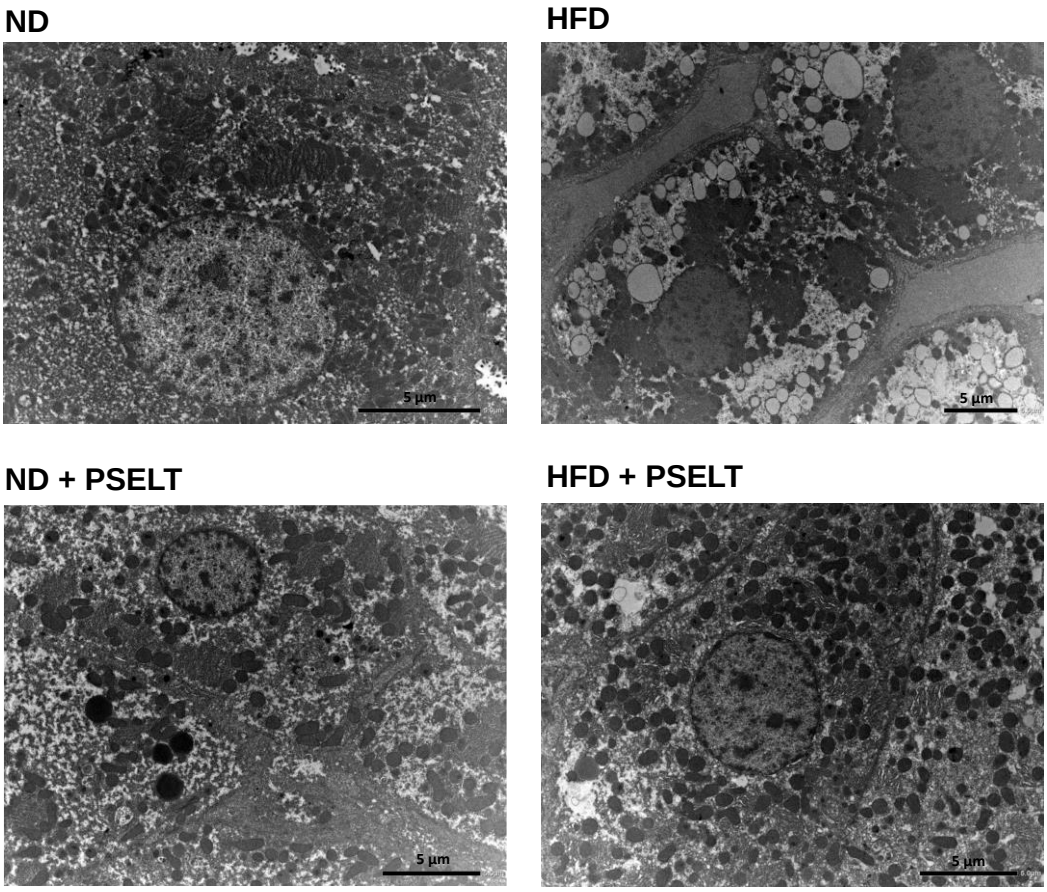


Figure 17. Effects of PSELT on hepatic structure in control and obese rats. *Ultrastructure appearance obtained by transmission electron microscopy (TEM) of hepatic sections ND, ND + PSELT, HFD, HFD + PSELT experimental groups. (Scale bar: 5 µm).*

1.8 PSELT action on endothelial integrity of abdominal aorta

Abdominal aorta integrity was then analysed in all experimental groups. Electron microscopy analysis revealed a conventional architecture of the endothelial cells of the abdominal aorta in both the control and animals treated with PSELT. Abdominal aorta of HDF rats showed necrosis and/or apoptosis of endothelial cells with their detachment from the underlying connective tissue (**Fig 18**). While, in HFD + PSELT group the endothelial cells of abdominal aorta exhibited a more preserved structure with normal attachment to the connective tissue (**Fig. 18**).

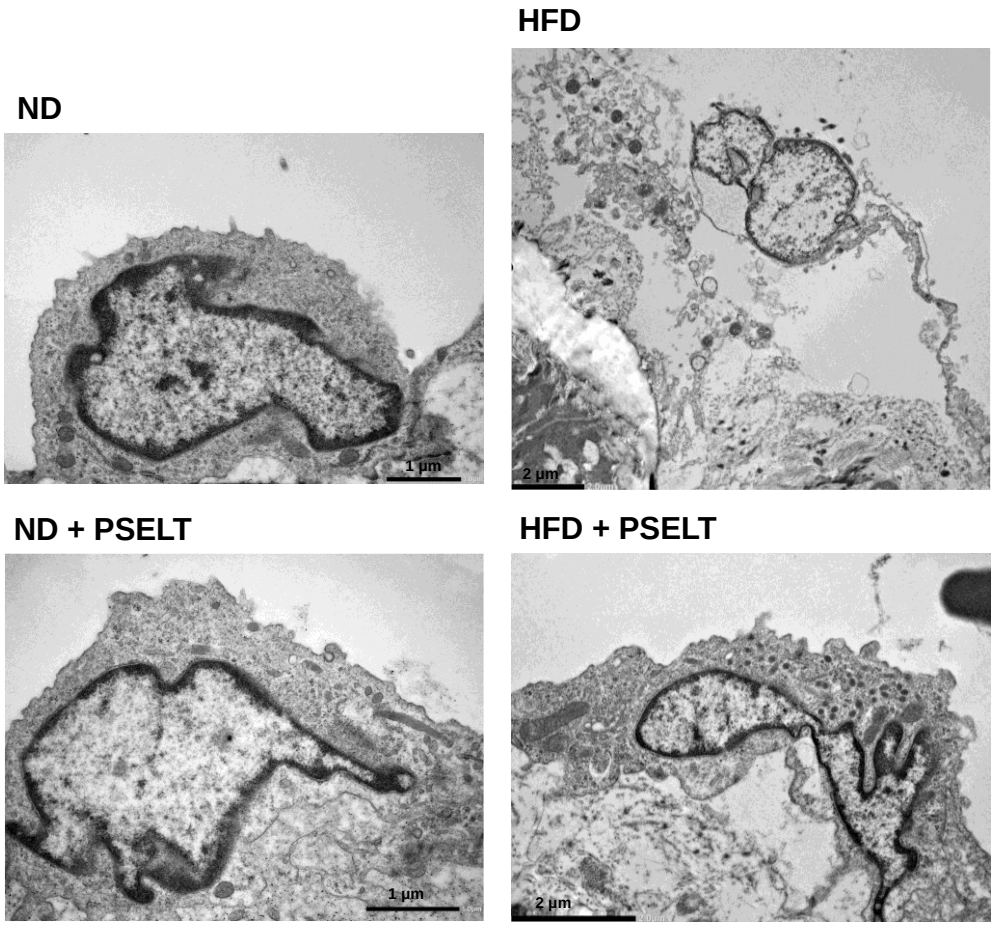


Figure 18. Effect of PSELT on abdominal aorta of control and obese rats. *Ultrastructure obtained by transmission electron microscopy (TEM) of the endothelium from abdominal aorta in ND, ND + PSELT, HFD, HFD + PSELT experimental groups. (Scale bar: 1 µm and scale bar: 2 µm).*

1.9 Effect of PSELT on BNP in LV myocardial tissues

The levels of BNP in LV myocardial tissues were measured at the end of the sacrifice. No significant increase of BNP levels was observed among the experimental groups fed with normal diet and treated with or without PSELT. While, BNP myocardial

concentration significantly augmented in HFD rats compared to the control animals. Furthermore, our data showed a significant reduction in BNP levels in HFD + PSELT group with respect to HFD alone (**Fig. 19**).

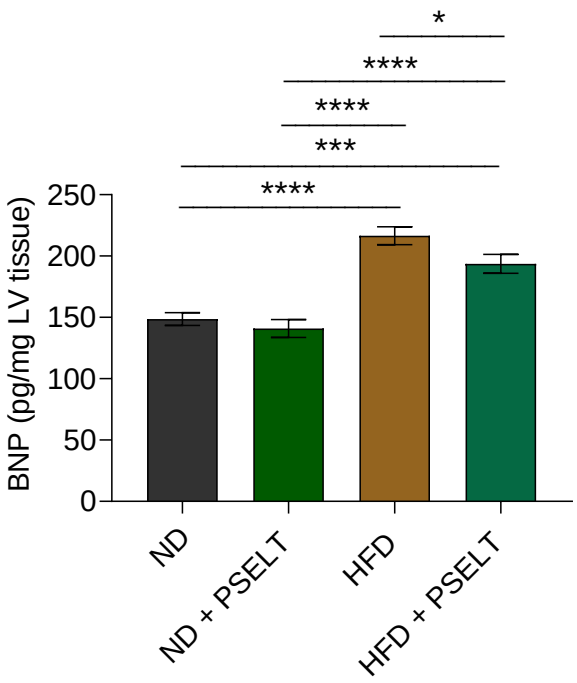


Figure 19. Effect of PSELT on BNP in left ventricular myocardial tissues. *Assessment of brain natriuretic peptide (BNP) in left ventricle of ND, ND + PSELT, HFD, HFD + PSELT (n = 6 for each experimental group). Data were expressed as the mean ± SEM. One-way ANOVA, Newman-Keuls multiple comparison was used. Statistically significant differences: *=p< 0.05, ***=p< 0.001, ****=p< 0.0001.*

2. Effect of PSELT in mice with diet-induced obesity

2.1 Effect of PSELT administrations on body weight and food intake

The effect of PSELT administration on weight gain in response to high fat diet dietary regimen was firstly evaluated. Starting from the 6th week of diet, a significant increase in body weight was observed in the animals fed with HFD compared to the ND group, confirming that the diet-induced obesity model has been established; while a significant decrease of weight has been observed in the animals fed with high fat diet and treated with PSELT (15 µg/kg) at the beginning of experimental protocol (indicated as "HFD + PSELT 16" group). Results showed that also in HFD fed mice treated with PSELT starting from the 9th week (indicated as "HFD + PSELT 8" group) the peptide was able to significantly decrease body weight compared to HFD fed mice (**Fig. 20 A**). Furthermore, food consumption measurements analysis showed a

significant reduction of food intake in both the HFD + PSELT 16 and HFD + PSELT 8 experimental groups compared to HFD group (**Fig. 20 B**).

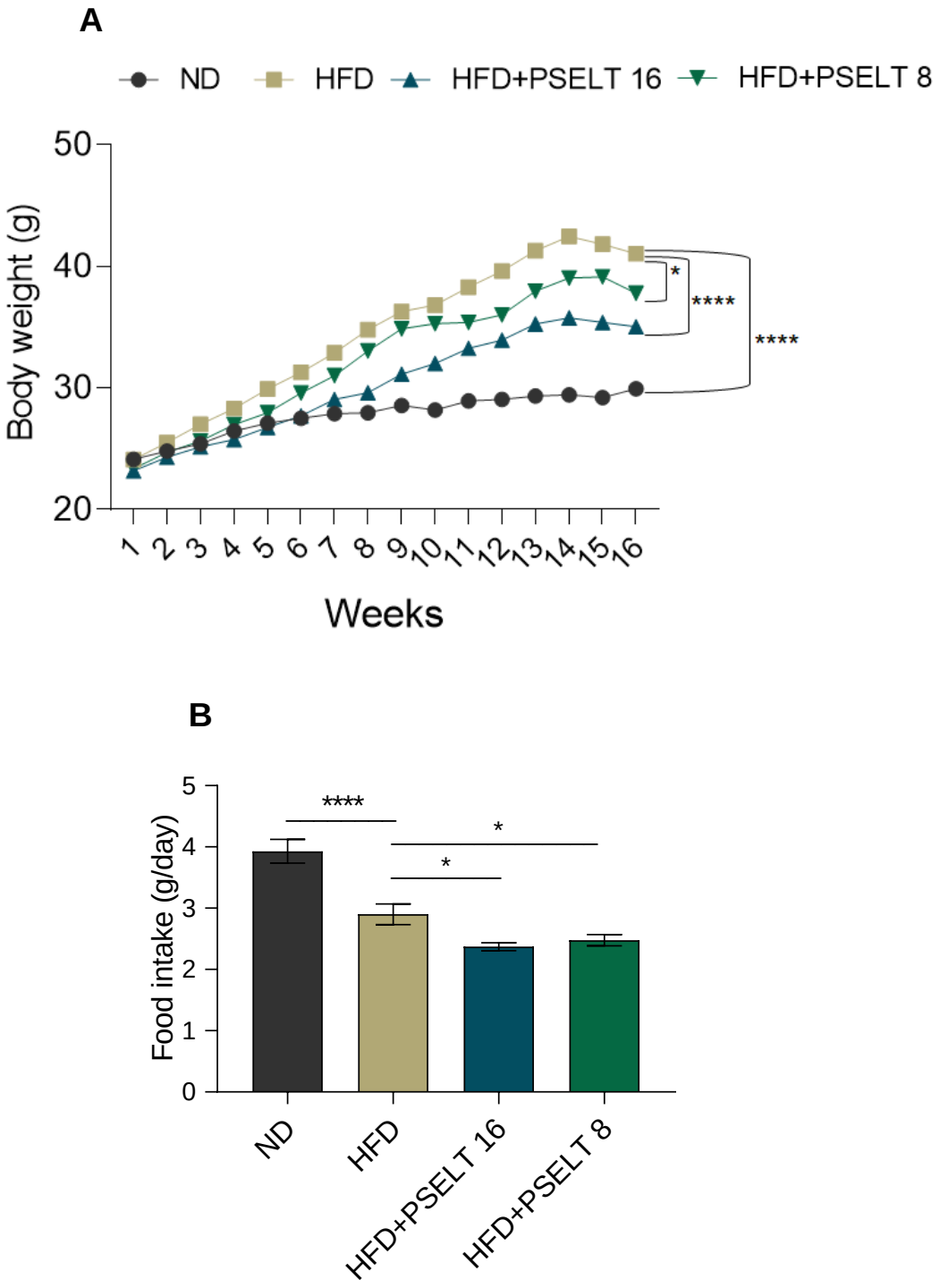


Figure 20. Influence of PSELT on body weight and food intake in mice models with diet-induced obesity A. *Body weight measurements* ***B.*** *Evaluation of food intake in ND, HFD, HFD +*

*PSELT 16, HFD + PSELT 8 (n=6 for each experimental group). Data were expressed as the mean ± SEM. Two-way ANOVA Bonferroni and One-way ANOVA (analysis of variance) Newman Keuls multiple comparison tests were used, respectively. Statistically significant differences: *=p < 0.05, ****=p< 0.0001.*

2.2 Assessment of body composition in control mice and in obese mice treated with or without PSELT

In order to establish whether the decrease in body weight and food intake in mice fed with HFD and exposed to PSELT could be linked to a reduction in fat mass and/or an increase in lean mass, a whole-body composition analysis was performed. Our results showed a significant increase in fat mass and fluids in all animals fed with HFD treated with or without PSELT compared to ND; a trend towards a reduction of fat mass, although not statistically significant, was observed only in HFD + PSELT 16 experimental group. Furthermore, a significant reduction of lean mass was displayed in HFD group with respect to ND group; however, a significant effect of both experimental HFD + PSELT 16 and HFD + PSELT 8 groups was not observed (**Fig. 21**).

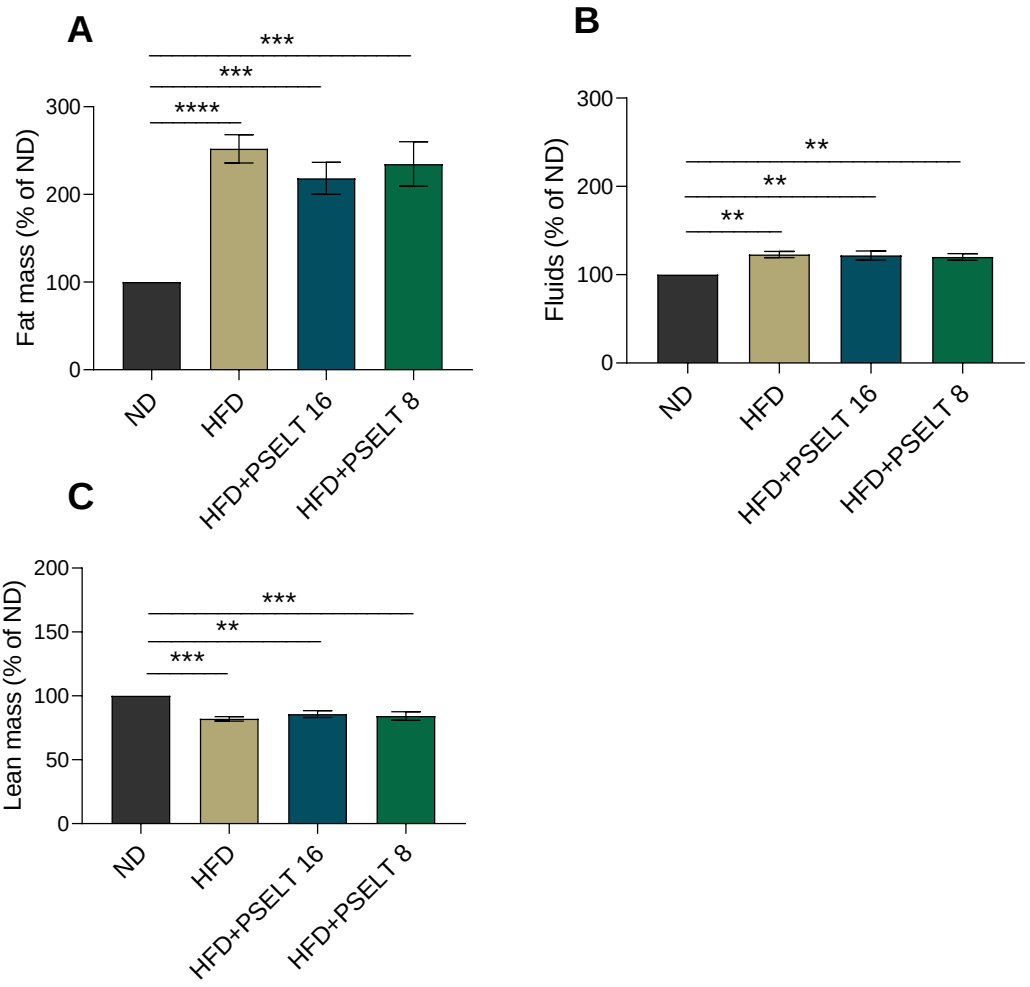


Figure 21. Effect of PSELT on body composition of control and obese mice. A. *Percentage of fat mass* ***B.*** *Percentage of fluids* ***C.*** *Percentage of lean mass in ND, HFD, HFD + PSELT 16, HFD + PSELT 8. (n=6 for each experimental group). Data were expressed as the mean ± SEM. One-way ANOVA (analysis of variance) Bonferroni post hoc was used. Statistically significant differences: **=p < 0.01, ***=p< 0.001, ****=p< 0.0001.*

2.3 Influence of PSELT on glycemia

To investigate the effect of PSELT in glucose metabolism, glycemia was monitored in the last 4 weeks of treatment in physiological fasted mice. Data showed higher blood glucose levels in HFD compared to ND group, however a significant reduction of glycemia was observed in both the experimental groups treated with PSELT compared with HFD group (**Fig. 22**).

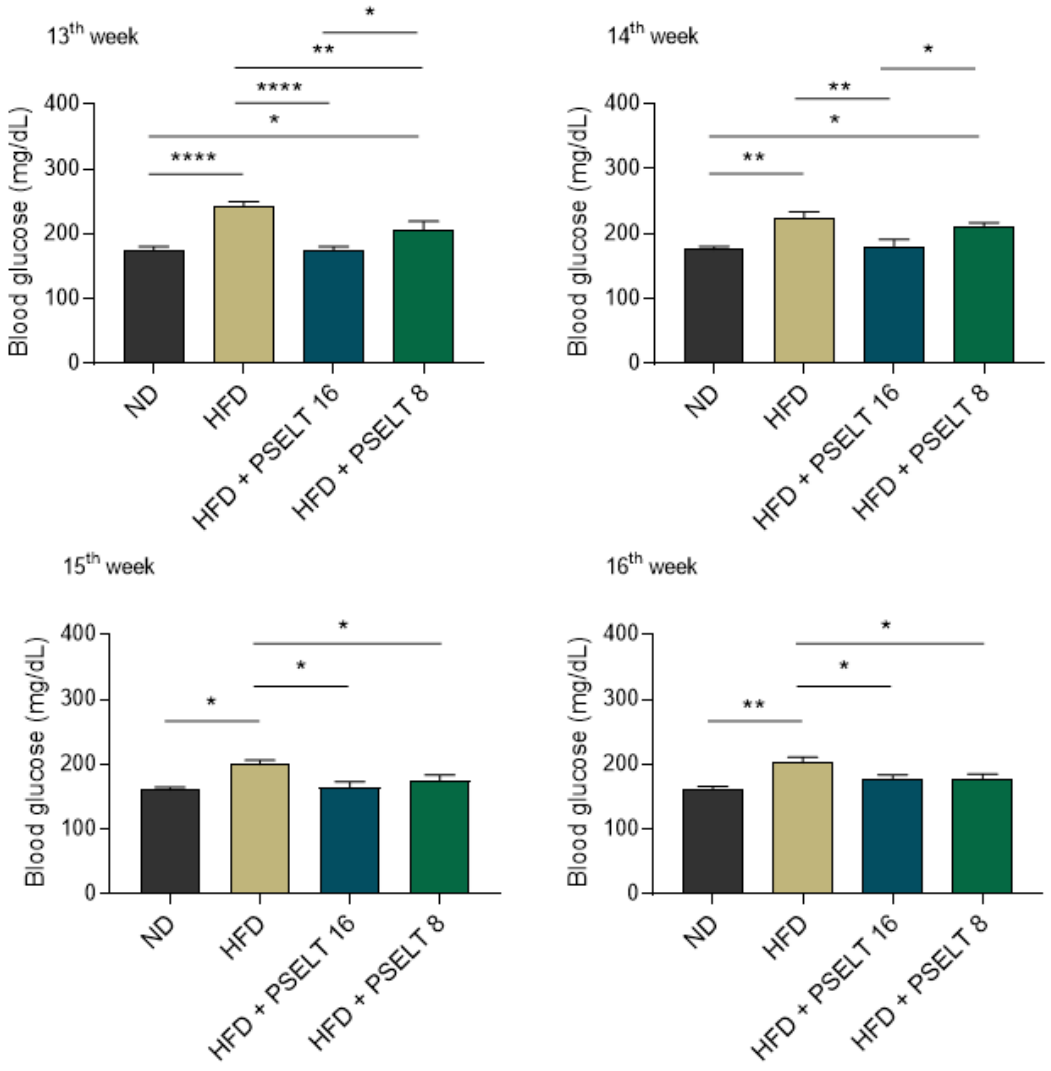
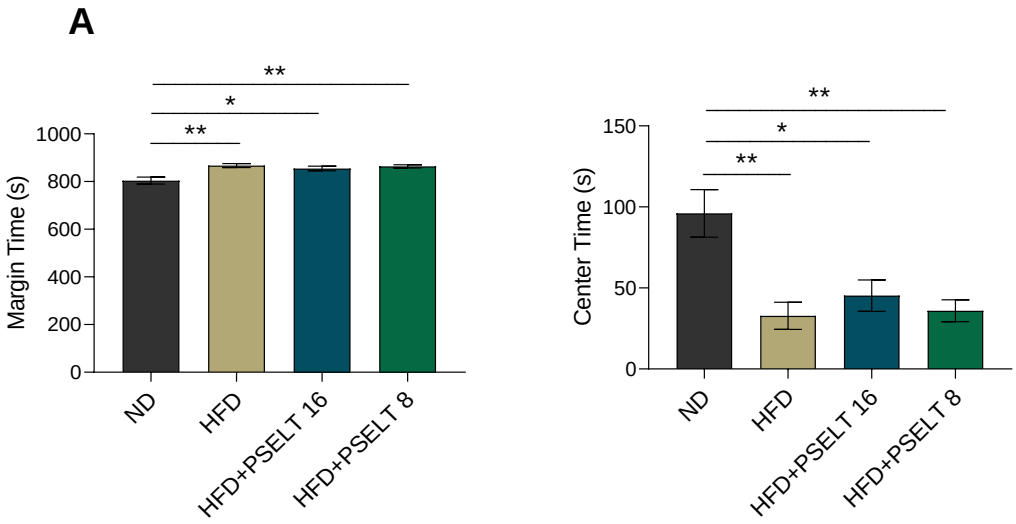
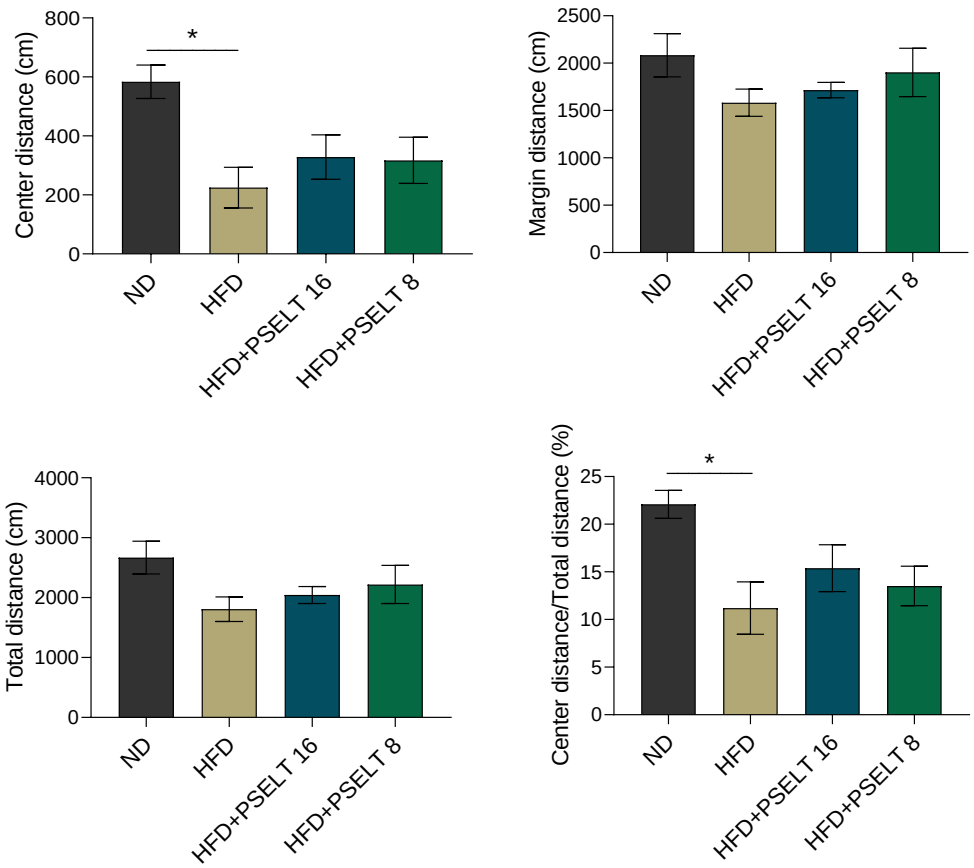


Figure 22. Action of PSELT on blood glucose levels in control and obese mice. *Glycemia measurements after 6 hours of fasting in mice in ND, HFD, HFD + PSELT 16, HFD + PSELT 8. (n=6 for each experimental group). Data were expressed as the mean ± SEM. One-way ANOVA (analysis of variance) Newman-Keuls. Statistically significant differences: *=p < 0.05, **=p < 0.01.*

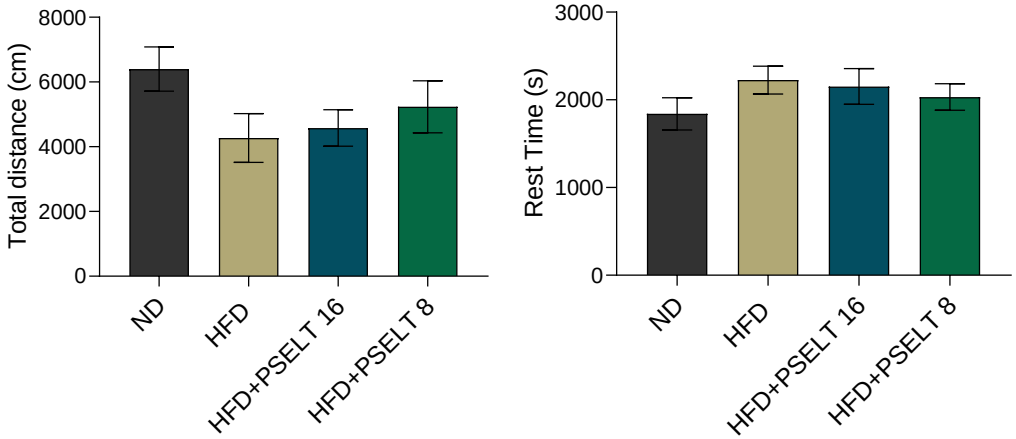
2.4 Effect of PSELT on anxiety-like phenotype and locomotor activity in normal and obese mice

Behavioural analyses were carried out in order to assess whether PSELT could mitigate anxiety-like behaviour induced by HFD treatment. Data deriving from open field analyses showed that the mice fed with HFD spent less time in the central area than control mice and more time in the marginal area of the apparatus compared to the control mice, indicating that HFD mice exhibited an anxiety-like phenotype. Despite no significant difference were revealed between HFD group and HFD + PSELT 16 or HFD + PSELT 8 groups, a trend toward an increase of center distance, margin distance, total distance and central distance/total distance was observed in mice treated with PSELT during HFD regimen (**Fig. 23 A**). Furthermore, from locomotor activity analysis no significant differences were found between ND and all HFD experimental groups (**Fig. 23 B**).




Center distance (cm)
800
600
400
200
0
*
ND
HFD
HFD+PSELT 16
HFD+PSELT 8
Margin distance (cm)
2500
2000
1500
1000
500
0
Total distance (cm)
4000
3000
2000
1000
0
Center distance/Total distance (%)
25
20
15
10
5
0

B


Total distance (cm)
8000
6000
4000
2000
0
ND
HFD
HFD+PSELT 16
HFD+PSELT 8
Rest Time (s)
3000
2000
1000
0

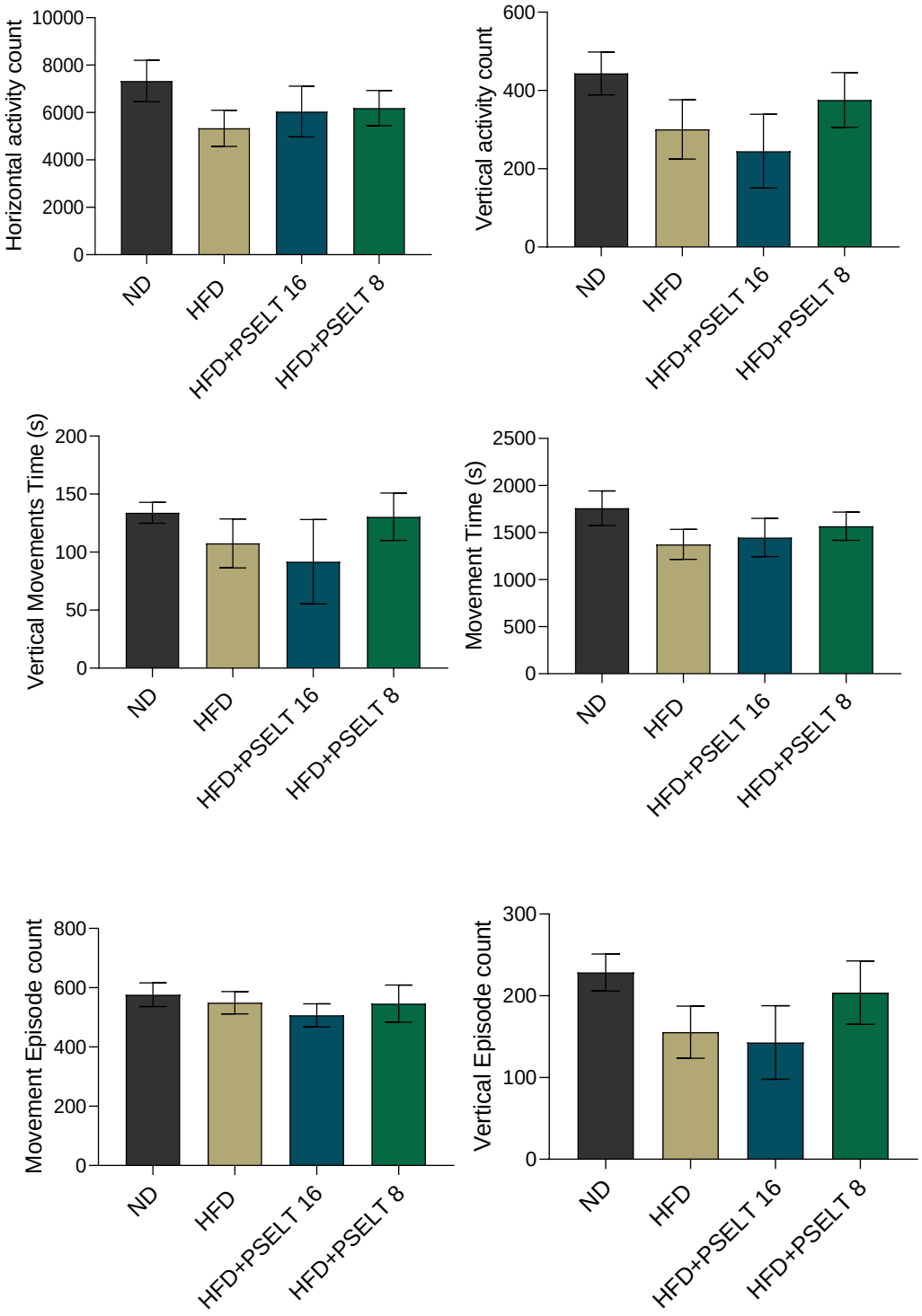


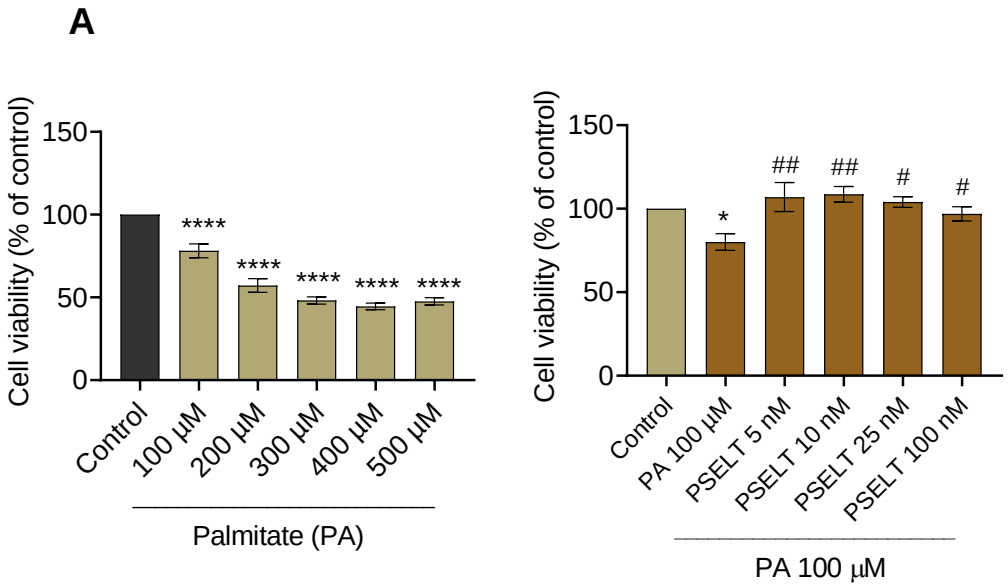
Figure 23. Influence of PSELT on anxiety-like behaviour (assessed by open field test) and locomotory activity. A. *Margin time (s), Center time (s), Center distance (cm), Margin distance (cm), Total distance (cm), Center time/Total distance (%) in ND, HFD, HFD+PSELT 16, HFD+PSELT 8, n=6 mice for each experimental group. Data were expressed as the mean ± SEM. One-way ANOVA (analysis of variance) Bonferroni post hoc was used. Statistically significant differences: *=p < 0.05, **=p < 0.01.* ***B.*** *Total distance (cm), Rest time (s), Horizontal activity count, Vertical activity count, Vertical movements time (s), Movement time (s), Movement*

Episode count, Vertical Episode count in ND, HFD, HFD + PSELT 16, HFD + PSELT 8, n=6 mice for each experimental group. Data were expressed as the mean ± SEM. One-way ANOVA (analysis of variance) Bonferroni post hoc was used.

3. Effect of PSELT against lipotoxicity and oxidative stress *in vitro*

3.1 Effect of PSELT on palmitate induced-lipotoxicity in H9c2 cells

In order to evaluate the protective action of PSELT against cardiac lipotoxic injury, palmitate (PA) was used for establishing a lipotoxicity model in H9c2 cardiomyocytes. Firstly, cells were treated with increasing concentrations of palmitate for 24 h and cell viability was evaluated. MTT analysis showed a significant reduction in H9c2 cell viability starting from the first concentration of palmitate tested (**Fig. 24 A)**. Subsequently, cell viability was evaluated in presence or absent of PSELT in H9c2 also exposed to palmitate. Our data showed a significant decrease in cell viability in palmitate-treated cells, however a significant increase in H9c2 cell viability was observed in palmitate-PSELT cotreated cells (**Fig. 24 A**). Results related to Oil Red O staining also displayed a gradual increase in terms of lipid accumulation in H9c2 cells after treatment with PA at 100 μM and 200 μM concentrations, indicating that a lipotoxicity *in vitro* model was established. In addition, PSELT treatment was able to mitigate palmitate- induced lipid accumulation in H9c2 cardiomyocytes (**Fig 24 B**).



B

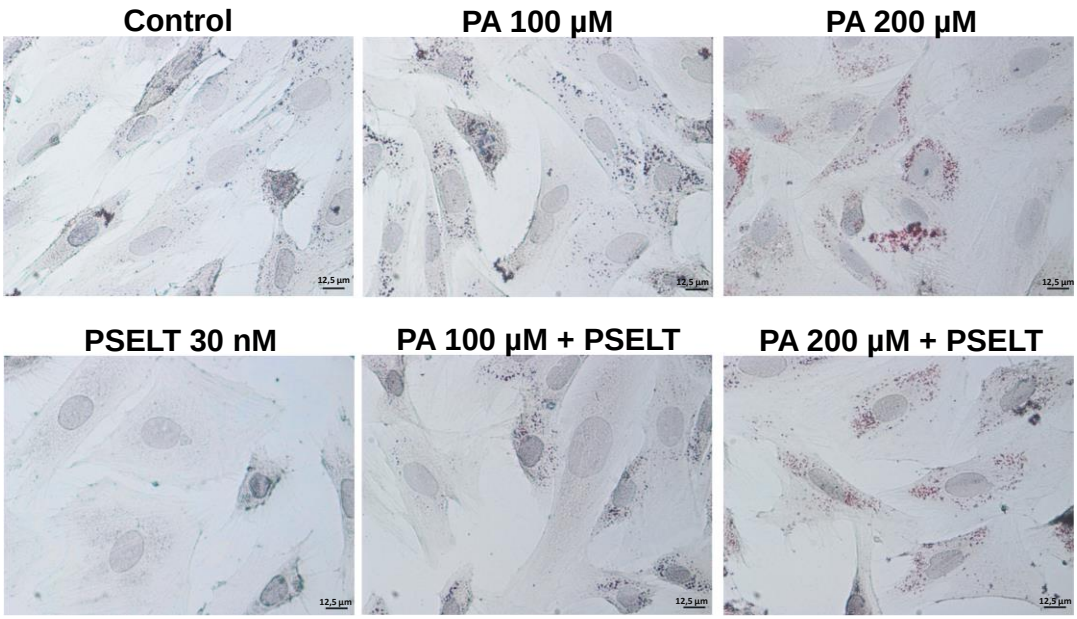
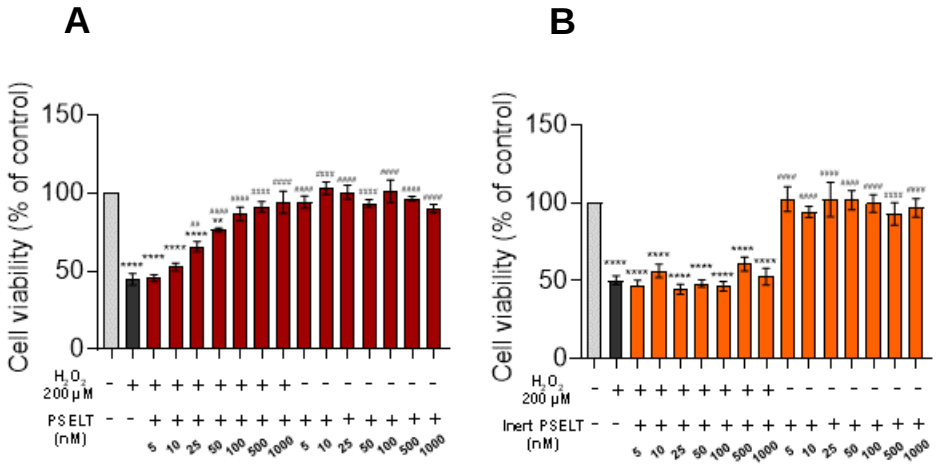


Figure 24. Action of PSELT in palmitate (PA)- induced lipotoxic effects on H9c2 cells. A. *H9c2 cells treated with increasing concentration of palmitate (from 100–500 µM) for 24 h and H9c2 cotreated with palmitate and increasing concentrations of PSELT for 24 h. Cell viability was evaluated by using MTT assay and was expressed as the percentage of control cells. Data are represented as mean ± SEM (n = 6 per group). Significant differences were detected by one-way ANOVA, Dunnett's multiple comparison test and Newman-Keuls multiple comparison test as post hoc were used, respectively. *p < 0.05, ****p < 0.0001 vs Control, #p < 0.05, ##p < 0.01 vs PA.* ***B.*** *H9c2 cells co-treated with palmitate and PSELT for 24 h and then stained by Oil Red O. Lipid droplets in cardiomyocytes were stained red. Scale bar, 12.5 µm.*

3.2 Effect of PSELT on hydrogen peroxide induced-oxidative stress in H9c2 cells

In order to evaluate the beneficial action of PSELT against a direct oxidative damage, hydrogen peroxide (H_2O_2) was employed for establishing an oxidative stress model in H9c2 cells. Cardiomyocytes were pre-treated with increasing concentrations of PSELT or *inert* PSELT for 24 h and then exposed to H_2O_2 (200 µM) for 3 h, and cell viability was evaluated. MTT assay showed a significant reduction in H9c2 cell viability after H_2O_2 treatment; however, PSELT was able to significantly mitigate H_2O_2-dependent cell death in a dose-dependent manner (**Fig. 25 A**). In particular,

PSELT was active starting from 25 nM reaching the maximum effect at 1000 nM. The same experiment was carried out by using *inert* PSELT, that did not exert any significant action on the reduction of cell viability secondary to H_2O_2 exposure (**Fig. 25 B).** Based on these results, we performed SELENOT protein expression analyses in order to establish whether the protective effects observed in PSELT pre-treated cells were also attributable to the endogenous protein action. Our data showed a significant reduction of SELENOT expression in H_2O_2 treated cells compared to control cells, while a significant increase in SELENOT, markedly evident at higher PSELT concentrations, was observed in PSELT pre-treated cells and then exposed to H_2O_2 (**Fig. 25 C**). Subsequently, we evaluated the effect of SELENOT *knockdown* by a SELENOT small interfering RNA (siRNA) on PSELT-induced protective effects in H9c2 cells after H_2O_2 exposure. To this aim, we firstly assessed the efficiency of SELENOT *knockdown* in H9c2 cells transfected with siRNA for SELENOT (si-SELENOT). Western blot and densitometric analysis showed in **Fig. 25 D** indicate that SELENOT expression levels were significantly decreased in si-SELENOT group compared with control group. Then, si-SELENOT transfected cells were pre-treated with increasing concentrations of PSELT (5, 10, 25, 50, 100, 500 and 1000 nM) for 24 h before H_2O_2 exposure. Our results showed a significant decrease of cell viability in si-SELENOT transfected cells compared with control cells (si-NC) and that H_2O_2 exposure, with or without si-SELENOT, further decreased cell viability respect to si-NC control group (**Fig. 25 E**). Data also shows that PSELT (5, 10, 25, 50, 100, 500 and 1000 nM) administration in si-SELENOT transfected H9c2 cells exposed to H_2O_2 partially recovered cell viability, although only at 500 nM was statistically significant, compared with si-SELENOT H9c2 cells treated with H_2O_2.



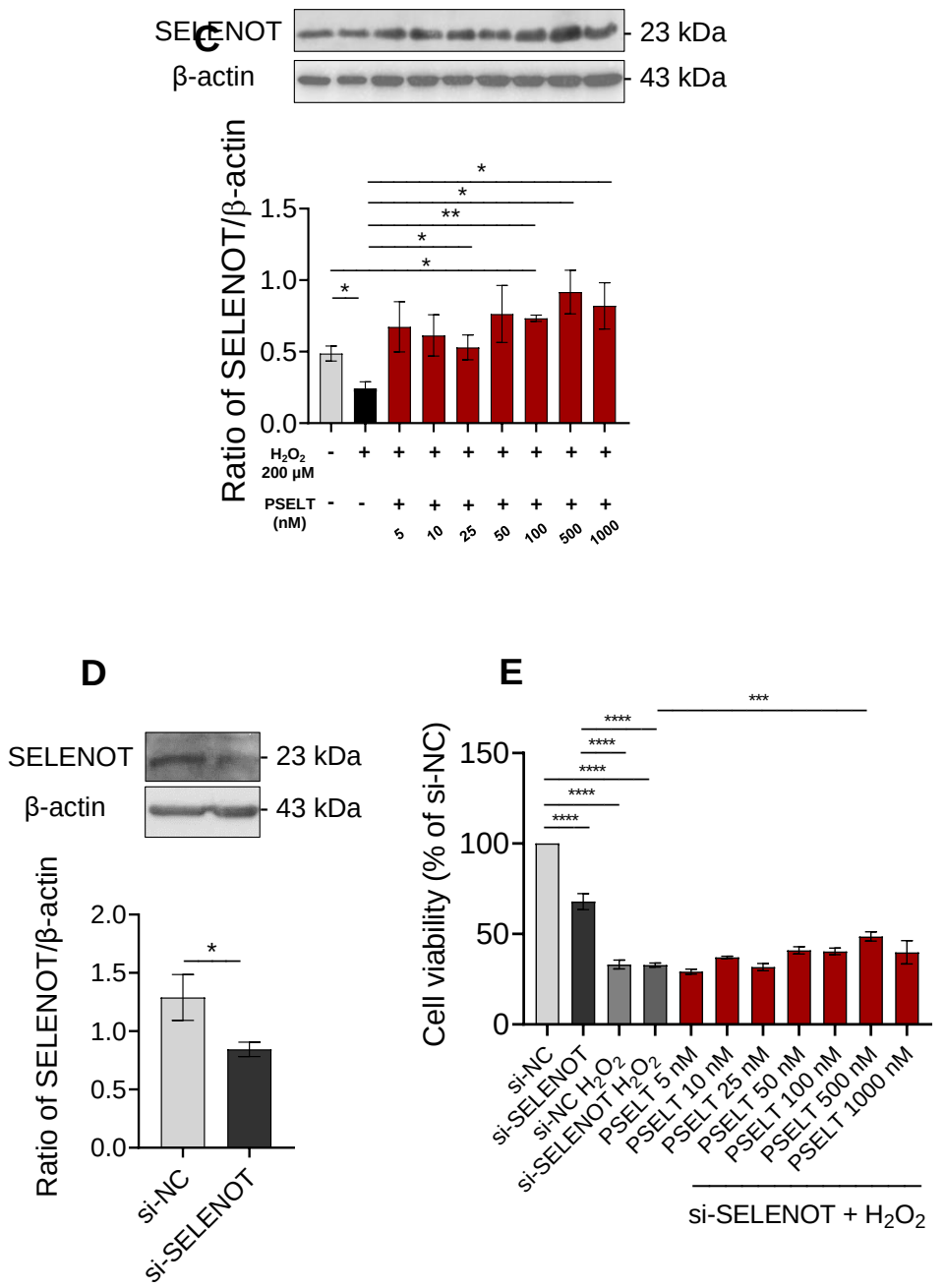


Figure 25. Effects of PSELT in the presence of hydrogen peroxide (H_2O_2) on H9c2 cell viability. A. *H9c2 cells pretreated with PSELT (from 5–1000 nM) for 24 h and then exposed to hydrogen peroxide (H_2O_2 200 µM) for 3 h* ***B.*** *H9c2 cardiomyocytes pre-treated with increasing concentrations of inert PSELT for 24 h and then exposed to H_2O_2 for 3 h. Cell viability was evaluated by using MTT assay and was expressed as the percentage of control cells. Results are represented as mean ± SEM (n = 6 per group). Significant differences were detected by one-way ANOVA and Newman-Keuls multiple comparison test. ****p < 0.0001 vs Control, ##p < 0.01, ###p < 0.001, ####p < 0.0001 vs H_2O_2.* ***C.*** *Western blot analyses of SELENOT and β-actin in H9c2 cardiomyocytes pre-treated with increasing concentrations of PSELT for 24 h and then exposed to H_2O_2 for 3 h. Histograms represent the ratio of densitometric analysis of protein:loading control. Significant differences were detected by Student's t test: p < 0.05 (*); p < 0.01 (**).* ***D.***

Representative image of SELENOT knockdown in H9c2 cardiomyocytes, transfected with 100 nM siRNA-SELENOT or negative control (siRNA-NC). Data were shown as mean ± SEM from three independent experiments. Histograms represent the ratio of densitometric analysis of protein:loading control. Significant differences were detected by t-test. $p < 0.05$ (*) vs si-NC group. ***E.*** *Effect of si-SELENOT gene silencing on cell viability in H9c2 cells pre-treated with increasing concentration of PSELT for 24 hours and exposed to H_2O_2 for additional 3 hours. Cell viability was evaluated using MTT assay and was expressed as the percentage of control cells only transfected with siRNA negative control (indicated as si-NC). Results are represented as mean ± SEM (n = 6 per group). Significant differences were detected by one-way ANOVA followed by Newman-Keuls multiple comparison test, $p < 0.001$ (***) and $p < 0.0001$ (****).*

Discussion

In the present doctoral thesis, we sought to examine the metabolic and cardiac beneficial effects of a specific Selenoprotein T-derived peptide ($SELENOT_{43-52}$ indicated as PSELT), encompassing the active catalytic site (CysValSerSec) of the *full-length* protein, in rat and mouse models of diet-induced obesity; the project also investigated whether and to which extend PSELT could protect cardiomyocytes against direct oxidative damage and lipotoxicity *in vitro*.

1. Effects of PSELT in obese rats

It is widely known that a high fat-based regimen is responsible for typical alterations of the balance between calories intake and their consumption resulting in substantial changes of body weight with consequent development of overweight and obesity (*Kopelman PG, 2000*). Diets with elevated fat content not only induce obesity in humans but also in animal models, in particular in rats and mice, making rodents suitable models for studying the pathogenesis of obesity and their complications (*Hariri & Thibault, 2010*; *Kim et al., 2016*; *Della Vedova et al., 2016*). According to our previous studies (*Casacchia et al., 2019*; *Nettore et al., 2019*), rats submitted to HFD (60% Kcal from fat) for 12 weeks develop an obese phenotype, as evinced by an increase in body weight compared to rats fed with normal diet (6.2 % Kcal from fat), and a decrease in food intake due to higher caloric content and a significant increase in BMI. Therefore, in the present study, we used the same diet regimen to induce obesity, and we tested the anti-obesogenic and cardioprotective action of PSELT by daily administering its physio-pharmacological doses (15 μg/kg/day) starting from the nineth week of the diet (for 28 days until the end of the dietary regimen), time at which animals begin to develop severe obesity and related complications, such as cardiac alterations and metabolic syndrome (*Casacchia et al., 2019*; *Nettore et al., 2019*). This experimental setting aimed to emulate a pharmacologically-based regime for treating obesity. We firstly found that, compared to rats fed with HFD only, animals fed with HFD and treated with the peptide exhibited a reduction of the weight gain and BMI, without alteration in food consumption. Although the reduction of body weight and BMI was significantly different between HFD+PSELT group compared to HFD, the effect of PSELT in controlling "anthropometric" parameters is interesting, particularly in regard to the fact that PSELT was administered only in the last 28 days of diet.

It has been widely demonstrated that obesity induces lipid accumulation at visceral and subcutaneous levels (*Matias et al., 2018*) and lipid deposition (ectopic fat) in non-adipose tissues, such as the liver, can also be observed (*Turpin et al., 2009*). In our obese rat model, we observed a significant increase in fat deposition, in

particular at the abdominal, perirenal, epididymal and retroperitoneal levels; notably, all these types of fat were significantly reduced in obese rats treated with PSELT. Since the intra-abdominal visceral deposition of adipose tissue is mainly responsible for the development of hypertension, increased insulin levels and insulin resistance, as well as diabetes mellitus and dyslipidaemia (*Kopelman 2000*), our data is of particular interest. Accordingly, several studies showed that prolonged HFD in rats induces typical alterations such as hyperglycaemia, insulin resistance and hypertriglyceridemia, leading to the clinical manifestations underlying human obesity and metabolic syndrome (*Buettner et al., 2007*; *Kraegen et al., 1991*; *Kelley et al., 1996*). In particular, the insulin resistance in the present thesis was assessed using a tolerance test for glucose (IPGTT); in this regard, we firstly observed that obese rats showed a significant increase in baseline glycaemia measured in the last week of the diet and a reduced glucose tolerance, while these effects were significantly reversed in obese rats treated with PSELT, indicating the ability of the peptide to improve insulin sensitivity and glycaemic profile. This result is of particular interest since hyperglycaemia also contributes to alteration of cardiac structure and function through advanced glycation end-products and posttranslational modification of extracellular matrix proteins, contributing to systolic and diastolic cardiac dysfunction (*Turer et al., 2012*). Our findings are further supported by emerging data indicating that selenium and selenoproteins, including SELENOT, are crucially implicated in the regulation of glucose homeostasis. For instance, a study conducted by Prevost and colleagues (*Prevost et al., 2013*) provided the first direct evidence regarding the crucial involvement of SELENOT in the control of glucose homeostasis. The authors showed that islets from conditional pancreatic β-cell SELENOT-*knockout* mice (SELENOT-insKO) were smaller and more numerous compared with islets from their wild-type littermates; in addition, SELENOT-insKO mice had higher blood glucose levels than wild-type animals and display alterations in insulin production and secretion.

We also observed that the obesity-dependent alteration of lipid profile was significantly mitigated by PSELT treatment. In particular, HFD + PSELT-treated animals displayed decreased plasma levels of total cholesterol, LDL cholesterol and triglycerides, and concomitantly higher HDL levels with respect to the HFD rats.

Taken together, these results indicate that PSELT may globally reduce the cardiovascular risk due to the alteration of plasma glucose and lipid profiles associated with obesity (*Klop et al., 2013*).

It has been reported that obese patients are prone to develop heart failure with preserved ejection fraction (*Yancy et al., 2013*), with cardiac hypertrophy and fibrosis. In our study, we found that HFD regimen induces a significant increase in the cardiac ventricular levels of the brain natriuretic peptide (BNP) compared to the control ND animals, and that PSELT significantly decreases this parameter. When released into the bloodstream, BNP is considered a diagnostic and prognostic marker for hypertrophy and heart failure and is extensively employed in clinic and biomedical research (*Sergeeva & Christoffels, 2013*) It is therefore conceivable that PSELT could reduce the increased susceptibility of HFD rats to develop heart failure.

Since obesity presents an augmented risk in the development of non-alcoholic fatty liver disease (NAFLD), a condition that can inevitably progress towards cirrhosis and liver failure (*Neuschwander-Tetri, 2007*; *Saadeh, 2007*; *Fabbrini et al., 2010*), we investigated the PSELT-dependent effect on the hepatic structure. Data deriving from transmission electron microscopy (TEM) analysis showed profound alterations of hepatic microarchitecture in rats exposed to hyperlipidic diet, with lipid accumulation and steatosis. Conversely, these manifestations were remarkably mitigated by PSELT, indicating that the peptide is able to preserve the liver structure and inhibit lipid accumulation. Notably, the beneficial action of SELENOT during liver injury has been addressed, at least in part, by Tanguy et al., (2011) (*Tanguy et al., 2011*). The authors found that SELENOT expression is strongly stimulated in liver cells during the regenerative process that occurs after partial hepatectomy in rats; although the role of this protein in the regenerating, as well as in injured liver, is not completely understood, our data provides further information related to the role of SELENOT as a cytoprotective agent during liver dysfunction.

Several studies report the close relationship between obesity and endothelial dysfunction (*Pierce et al., 2008*; *Burke et al., 2008*; *Al Suwaidi et al., 2001*), which plays a critical role in the onset and progression of cardiovascular disease. It is also known that chronic low-grade inflammation occurring during obesity can augment ROS and reduce NO production in endothelial cells (*Serpillon et al., 2009*; *Kobayasi et al., 2010*; *Santilli et al., 2015*). This prompted us to hypothesize that PSELT could counteract the endothelial alteration secondary to HFD regimen. For this purpose, we performed TEM analysis of abdominal aorta endothelium sections. The ultrastructural alterations, particularly evidenced by a detachment of the endothelial cells from connective tissue with consequent necrosis and/or apoptosis, observed in obese rats were mitigated by PSELT; this finding indicates that the peptide is also able to restore the endothelial integrity of aorta resulting in an

improvement of its normal architecture and protecting the aorta structure from the harmful effects of HFD. Endothelial dysfunction and aorta alterations represent significant predictors of increased cardiovascular disease mortality, as well as important risk factors for coronary artery disease. As described above, PSELT ameliorated the metabolic profile during HFD and this may be indicative of a reduced risk of myocardial infarction, as suggested by the direct relationship described between abdominal obesity and the increased risk of acute myocardial infarction (*Bays 2014*; *Zhu et al., 2014*). Therefore, we tested the cardioprotective effect of PSELT in rats following an *ex-vivo*-induced myocardial I/R injury at the end of chronic treatments. Consistent with this, the heart of rats fed with both ND or HFD and treated with PSELT showed a higher protection against I/R injury with respect to ND or HFD only animals, respectively. This was evidenced by a significant reduction of IS and by a marked improvement of the post-ischemic contractile function expressed as an increase in dLVP and a decrease of contracture development, measured by LVEDP. Data from the literature suggest that lipid accumulation within cardiac muscle may participate in the progression of heart failure and that an accumulation of triacylglycerols is associated with cardiac contractile dysfunction *(Stanley et al., 2005).* Our ultrastructural analyses revealed an accumulation of lipid droplets and lipofuscin granules in the hearts of obese rats, with myofibrillar degeneration; on the contrary, we observed a marked improvement of cardiac myocyte structure and a suppression of cytoplasmic accumulation of lipid droplets and lipofuscin granules in HFD rats treated with PSELT. Lipofuscin is an insoluble lipid pigment that in mammals accumulates in the lysosomal compartment of specific cell types like neurons, cardiac myocytes and skeletal muscle cells. Lipofuscin accumulation seems to have a detrimental effect on the performance and survival of myocardial cells (*Höhn & Grune, 2013*; *Terman et al., 2004*).

These results suggest that PSELT acts as a pharmacological pre-conditioning cardioprotector in both normal and obese conditions. Our findings are corroborated by recent data demonstrating the crucial role of SELENOT in cardiomyocyte differentiation and protection, as well as the cardioprotective action, as post-conditioning agent, of PSELT acting through the thioredoxin-like domain following myocardial I/R insult in rats (*Rocca et al., 2018*).

2. Action of PSELT in an obese murine model

A large body of evidence indicates that the consumption of HFD can lead to behavioural changes and increase the risk of neurodegenerative diseases.

Distinctive manifestations of these diseases include behavioural, motor and cognitive deficits due to a disruption of the biological actions exerted by neurotransmitters in the brain (*Han et al., 2021 and reference therein*). In order to study the role of PSELT in behavioural disorders induced by a high-fat diet, we used a mouse model of diet-induced obesity and we tested the action of the peptide as both preventive and treating agent against obesity. For this purpose, two experimental settings have been established; in the first experimental group (indicated as HFD + PSELT 16) PSELT was administered at the beginning of HFD, while in the second experimental group (indicated as HFD + PSELT 8) PSELT was administered starting from the ninth week of diet regimen. Based on our results, we firstly found a significant increase of body weight in HFD mice compared with control mice starting from 6^{th} week of diet, indicating that the obesity model was established, and a significant reduction in HFD + PSELT 16 mice with respect to HFD alone. A marked decrease of body weight was also observed in HFD + PSELT 8 experimental group starting from 12^{th} weeks of diet. Notably, in the case of the mouse model, we found that the reduction in body weight was linked to a significant decrease of food intake in both the experimental groups treated with PSELT, suggesting a central role of the peptide in food intake control. To evaluate whether the weight reduction induced by PSELT during HFD regimen could be linked to a reduction of fat mass and/or increase of lean mass, we performed body composition analysis. Our results showed that the peptide was not able to significantly mitigate the HFD-induced increase of fat mass and decrease of lean mass; however, this could be related to a small sample size used for this study. Indeed, higher sample size is normally used for body composition analysis.

Similar to what has been observed in the rat model, we found that also in both experimental mouse groups treated with PSELT, the peptide was able to significantly mitigate the hyperglycaemia secondary to HFD, corroborating the involvement of the peptide in glucose homeostasis.

Data from the literature reported that, while short-term consumption of a high-fat diet reduces anxiety, prolonged consumption promotes an anxiety-like phenotype. Furthermore, the insulin resistance developed in obese animals affects the regulation of emotional states and can enhance anxiety levels (*Sweeney et al., 2017 and reference therein*). Here, we examined the effect of the peptide on anxiety-like behaviours by open field; consistent with literature findings, obese mice showed a significant increase in the anxiety-like phenotype; however, we did not find

significant action of PSELT on the anxiety-related parameters, indicating that PSELT does not reduce the susceptibility to obesity-dependent anxiety.

3. Action of PSELT on palmitate-induced injury in H9c2 cardiomyocytes

It has been observed that diabetes and obesity are associated with lipotoxic cardiomyopathy that may be the result of a toxic metabolic shift to more fatty acid and less glucose oxidation with concomitant accumulation of toxic lipids (*Drosatos & Schulze, 2013*). In a context of high free fatty acid concentration, including palmitate, cardiac myocytes undergo apoptosis. Palmitate (PA), the most common long-chain saturated fatty acid, it is widely used in many cell types, including cardiomyocytes, to trigger apoptosis and myocardial damage following to the accumulation of lipid intermediates in non-adipose tissue and as inducer of lipotoxicity *in vitro*. The mechanisms underlying palmitate-induced lipotoxicity include endoplasmic reticulum stress and oxidative stress (*Kong & Rabkin, 2000 and reference therein*). We therefore employed palmitate for reproducing an *in vitro* model of cardiac alterations characterizing lipotoxicity and we tested the potential effect of PSELT in counteracting the myocardial damage induced by palmitate in H9c2 cells. We showed that PA significantly decreased cell viability compared to control cells and PSELT treatment was able to mitigate PA-dependent cell death. In addition, we found that PSELT reduced lipid droplets intracellular accumulation in H9c2 cardiomyocytes induced by PA treatment, as revelated by Oil Red O staining. Further studies are ongoing in order to elucidate the mechanism of action underlighted these results.

4. Effect of PSELT on oxidative damage induced by H_2O_2 in H9c2

Oxidative stress plays a major role in the pathophysiology of several cardiovascular diseases, such as myocardial I/R damage, atherosclerosis, hypertension and heart failure (*Forman & Zhang, 2021*). Existing evidence supports the view that antioxidant therapy may convey beneficial action in combating these problems. In this context, it has been demonstrated that SELENOT takes part to a crucial antioxidant program during neuroendocrine cell differentiation also contributing to maintain ER and redox homeostasis thanks to its antioxidant activity conferred by the Cys-Val-Ser-Sec sequence present in the thioredoxin-fold (*Anouar et al., 2018 and references therein*). In view of our recent findings demonstrating the antioxidant action of PSELT in the heart following I/R insult (*Rocca et al., 2018*) and the results obtained in the present study regarding the ability of the peptide to protect the heart

of both normal and obese rats, we hypothesized that PSELT could exert antioxidant action against oxidative damage in cardiomyocytes. To test this possibility, we employed H_2O_2 to trigger oxidative stress in H9c2 cells, a cell line that is widely used as a model of cardiomyocytes because of its biochemical, morphological and electrical/hormonal signalling properties (*Hescheler et al., 1991*; *Branco et al., 2015*). The results showed that pre-treatment with PSELT dose-dependently counteracted the detrimental action of H_2O_2 on cell viability, indicating that the peptide can protect cardiomyocytes from oxidative damage. Previous evidence indicated that H_2O_2 stimulates SELENOT gene expression to protect differentiating PC12 cells against oxidative stress (*Abid et al., 2019*). However, in our study we found that H_2O_2 exposure significantly decreased SELENOT expression compared with control cells; interestingly, increasing concentrations of exogenous PSELT were able to linearly rescue the protein expression, strongly upregulating it at higher doses (100-1000 nM). These data not only indicate that PSELT administration is able to stimulate SELENOT expression *per se* but also support the hypothesis that SELENOT may contribute, at least in part, to the peptide-dependent protection of cardiomyocytes against oxidative stress. To elucidate the potential synergic action of exogenous PSELT and endogenous SELENOT in protecting cardiomyocytes from oxidative damage, we evaluated the effect of SELENOT *knockdown* on PSELT-induced cytoprotection in H9c2 cardiomyocytes following H_2O_2 exposure. Our data indicates that the SELENOT deficiency negatively affected H9c2 cell viability treated with or without H_2O_2, indicating the essentiality of SELENOT in cardiomyocytes, and that the reduced viability by SELENOT knockdown was partially alleviated by exogenous PSELT, in accord with the idea that SELENOT plays a key role in the peptide antioxidant action and that the dual action of PSELT/SELENOT is required to confer cardioprotection.

Conclusions

In this doctoral thesis, we described for the first time the beneficial action exerted by a specific selenoprotein T-derived peptide, PSELT, in obese rat/mice models as well as on direct oxidative damage and lipotoxicity in H9c2 cardiomyocytes. Our results *in vivo*/*ex vivo* and *in vitro* provide important information about the crucial role exerted of the peptide at systemic and cardiac level both in dysmetabolic condition and stressfully condition. Notably, we showed that PSELT, while not acting on body composition, was able to counteract the high fat diet-induced weight gain by reducing food intake. The typical alterations occur during the MetS are linked to hyperglycemia, insulin resistance and dyslipidemia. We found that chronic

administration of PSELT restored glucose and lipid profile in obese animals, also improved insulin sensitivity. In addition, ultrastructural results further highlighted that PSELT was able to ameliorate cardiac, hepatic and endothelial alterations due to an excessive fat consumption, reducing ectopic lipid deposition. In line with its cardioprotective action as post-conditioning agent (Rocca et al., 2018), we also displayed that PSELT was capable to mitigate impaired basal hemodynamic parameters in obese rat heart, also improving post-ischemic recovery and decreasing the infarct size following I/R injury, by acting as pharmacological pre-conditioning agent. According with the antioxidant activity of the peptide, our *in vitro* findings suggest that PSELT protects cardiomyocytes against lipotoxic injury and H_2O_2-induced oxidative damage, also restoring the reduced SELENOT protein levels following pro-oxidant stimulus, and by acting together endogenous SELENOT in order to achieve cytoprotection and cardioprotection.

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Abbreviations

BMI: body mass index

FFAs: Free fatty acids

MetS: Metabolic syndrome

HDL: high-density lipoprotein cholesterol

CVDs: cardiovascular diseases

CHD: coronary heart disease

LDL: low-density lipoproteins

ROS: reactive oxygen species

NO: nitric oxide

eNOS: endothelial Nitric Oxide Synthase

TNF-α: tumor necrosis factor alpha

IL-1 β: interleukin-1beta

IL-6: interleukin-6

IFN -γ: interferon gamma

NF-κB: nuclear factor-kappa B

IR: ischemia/reperfusion

GPx1: glutathione peroxidase 1

Sec: Selenocysteine

TXNRDs: thioredoxin reductases

SELENOI: selenoprotein I

SELENOK: selenoprotein K

SELENOO: selenoprotein O

SELENOR: selenoprotein R

SELENOS: selenoprotein S

SELENOH: selenoprotein H

SELENOM: selenoprotein M

SELENON: selenoprotein N

SELENOT: selenoprotein T

SELENOV: selenoprotein V

SELENOW: selenoprotein W

SPS2: selenophosphate synthetase 2

Sep15: 15-kDa selenoprotein

Trx: thioredoxin

Cys: Cysteine

SELENOP: selenoprotein P

SELENOV: selenoprotein V

GPX: Glutathione peroxidase

GSH: Glutathione

GPx-SeOH: selenenic acid

GPx-SeSG: selenenyl sulfide

GPX2: Glutathione peroxidase 2

GPX3: Glutathione peroxidase 3

GPX4: Glutathione peroxidase 4

GPX5: Glutathione peroxidase 5

GPX6: Glutathione peroxidase 6

GPX7: Glutathione peroxidase 7

GPX8: Glutathione peroxidase 8

TXNRD: thioredoxin reductase

TXNRD1: thioredoxin reductase 1

TXNRD2: thioredoxin reductase 2

TXNRD3: thioredoxin reductase 3

H_2O_2: hydrogen peroxide

TRX1: Thioredoxin-1

DIOs: Iodothyronine Deiodinases

DIO1: Deiodinase type 1

DIO2: Deiodinase type 2

DIO3: Deiodinase type 3

T4: tetraiodothyronine

T3: 3,3',5-triiodothyronine

ER: endoplasmic reticulum

HepG2: human liver cancer cells

HEK 293: human embryonic kidney cells

HeLa: cervical cancer cells

SELENOM-KO: selenoprotein M knockout mice

CVSU: Cysteine-Valine-Serine-Selenocysteine

PACAP: pituitary adenylate cyclase-activating polypeptide

PC12: rat pheochromocytoma cells

cAMP: Cyclic adenosine monophosphate

UPR: unfolded protein response

SELENOT-insKO: conditional pancreatic β-cell SELENOT knockout mice

PD: Parkinson's diseases

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

ND: Normal Diet

HFD: High Fat Diet

IPGTT: intraperitoneal glucose tolerance test

KHs: Krebs-Henseleit solution

LV: left ventricle

CP: coronary pressure

dLVP: developed left ventricular pressure

LVEDP: left ventricular end-diastolic pressure

IS: Infarct Size

BNP: brain natriuretic peptide

TEM: transmission electron microscopy

H9c2: rat embryonic cardiomyocytes

ATCC: American Type Culture Collection

DMEM/F-12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

FBS: fetal bovine serum

MTT: 3-(4,5-dimethylthiazol-)2,5-diphenyl tetrazonium bromide

si-SELENOT: SELENOT small interfering RNA

si-NC: negative control siRNA

PA: Palmitate

DMSO: Dimethyl Sulfoxide

DPBS: Dulbecco's phosphate-buffered saline

University of Calabria-Italy, Laboratory of Cellular and Molecular Cardiovascular Pathophysiology, Dept. DiBEST

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Discipline: ***Physiology***

The Selenoprotein T-mimetic (PSELT) exerts cardiometabolic protection in rat and mouse models of obesity and metabolic syndrome

Ph.D. student

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Tutor

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*This study was supported by "**Vinci Project n. C2-1563**" Italian-French University*

Director of Doctorate in Life Sciences

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XXXIV Cycle of Doctorate in **Life Sciences**

Co-tutorship with the Normandy's Doctoral School of **Integrative Biology, Health and Environment** (EdN BISE 497)

Ph.D. student: Anna De Bartolo

Thesis: The Selenoprotein T-mimetic (PSELT) exerts cardiometabolic protection in rat and mouse models of obesity and metabolic syndrome

Research:

Dr. Anna De Bartolo has performed his Ph.D. scientific activity in the Laboratory of *Cellular and Molecular Cardiovascular Pathophysiology*, Department of Biology, Ecology and Earth Science, University of Calabria, and in the Laboratories of *Neuronal and Neuroendocrine Differentiation and Communication*, INSERM U1239, University of Rouen-France (from October 2019 to March 2020) in the context of the co-tutorship between the two Universities.The research was supported by the project of Italy-France cooperation "Vinci Project n. C2-1563".

In the Laboratory of *Cellular and Molecular Cardiovascular Pathophysiology* directed by Prof. Tommaso Angelone, Dr. De Bartolo studied, for the first time, the action of a selenoprotein T (SELENOT)-derived peptide, PSELT ($SELENOT_{43-52}$) in pathological condition related to metabolic disorder such as obesity and metabolic syndrome. Using *in vivo/ex vivo* and *in vitro* models, she investigated the protective effects of PSELT, chemically peptide synthetized in its reduced form, which encompasses the key redox motif of the *full-lenght* protein, at systemic and cardiac levels, also focusing about the role of the peptide against oxidative and lipotoxic cardiac damage.

Dr. De Bartolo found that PSELT is very effective in counteract impaired anthropometric and metabolic variables during obesity and metabolic syndrome, also acting on glucose and lipid metabolism, and resulting an improvement in glycemia and insulin sensitivity, as well as in lipid

profile and lipid deposition at adipose tissue level. She analyzed the effects of PSELT on obese rat heart under basal condition and after ischemia/reperfusion (I/R) injury, in terms of myocardial contractility and relaxation recovery, showing that PSELT elicited cardioprotection, acting as a pharmacological preconditioning agent. This is evidenced by a strong improvement of the systolic function at baseline and reduction of natriuretic peptide type B (BNP) levels (prognostic marker of heart failure) at left ventricle level, as well as significant recovery of contractility (developed Left Ventricular Pressure, dLVP) and a reduction of infarct size (IS), without changes in cardiac contracture (Left Ventricular Endo-Diastolic Pressure, LVEDP) following by I/R protocols. By using transmission electron microscopy (TEM), Dr. De Bartolo showed that PSELT protects the heart counteracting lipid accumulation and myofibrillar degeneration, improves hepatic ultrastructure reducing steatosis, and mitigates endothelial dysfunction though inhibition of apoptosis/necrosis in endothelial cells of abdominal aorta. By using an in vitro model of cardiomyocytes (H9c2 cells), Dr. De Bartolo showed that PSELT exerts protective action on cardiomyocytes against a direct oxidative damage and lipotoxic injury, by a mechanism that involved SELENOT contribution.

During her stage at the Laboratories of Neuronal and Neuroendocrine Differentiation and Communication, directed by Dr. Youssef Anouar, Dr. De Bartolo also performed experiments in a murine model of diet-induced obesity, in order to establish the action of PSELT in behavioral disorders related to anxiety and locomotor activity. Dr. De Bartolo found that PSELT significantly counteracts weight gain due to high fat diet consumption reducing food intake, and attenuates hyperglycemia that occurs during obesity, corroborating the results obtained in obese rat model. In addition, she found that PSELT chronic administrations tend toward reduce the obesity-dependent anxiety-like phenotype, however her results did not shown changes about locomotor activity.

During her Ph.D. activity, Dr. De Bartolo has attended seminaries and training courses in Italy, in the context of the Doctorate in Life Sciences. She has been tutor of several students of Biology and Pharmacy degree courses during their thesis preparation and support teacher for Physiology and Neurophysiology courses.

During her Ph.D. period, Dr. De Bartolo obtained important new and publishable data that are illustrated in her thesis. Part of her work will be shortly published on an International Journal.

Academic College exhibits a positive opinion for the excellent research work conducted by Dr. Anna De Bartolo.

Published

1. Alam W, Rocca C, Khan H, Hussain Y, Aschner M, **De Bartolo A**, Amodio N, Angelone T, Cheang WS. Current Status and Future Perspectives on Therapeutic Potential of Apigenin: Focus on Metabolic-Syndrome-Dependent Organ Dysfunction. *Antioxidants* 2021, 10, 1643. https://doi.org/10.3390/antiox10101643.

2. Grande F*, **De Bartolo A***, Occhiuzzi MA, Caruso A, Rocca C, Pasqua T, Carocci A, Rago V, Angelone T, Sinicropi MS. Carbazole and Simplified Derivatives: Novel Tools toward β-Adrenergic Receptors Targeting. *Applied Sciences*. 2021; 11(12):5486. https://doi.org/10.3390/app11125486. ***Equally contributed**

3. Rocca C*, **De Bartolo A*,** Grande F, Rizzuti B, Pasqua T, Giordano F, Granieri MC, Occhiuzzi MA, Garofalo A, Amodio N, Cerra MC, Schneider F, Panno ML, Metz-Boutigue MH, Angelone T. Cateslytin abrogates lipopolysaccharide-induced cardiomyocyte injury by reducing inflammation and oxidative stress through toll like receptor 4 interaction. *International Immunopharmacology*. Febbraio 2021. https://doi.org/10.1016/j.intimp.2021.107487. ***Equally contributed**

4. Rocca C, Grande F, Granieri MC, Colombo B, **De Bartolo A**, Giordano F, Rago V, Amodio N, Tota B, Cerra MC, Rizzuti B, Corti A, Angelone T, Pasqua T. The chromogranin A1-373 fragment reveals how a single change in the protein sequence exerts strong cardioregulatory effects by engaging neuropilin-1. *Acta Phisyologica*. https://doi.org/10.1111/apha.13570.

5. Pasqua T, Rocca C, Lupi FR, Baldino N, Amelio D, Parisi OI, Granieri MC, **De Bartolo A**, Lauria A, Dattilo M, Perrotta ID, Puoci F, Cerra MC, Gabriele D, Angelone T. Cardiac and Metabolic Impact of Functional Foods with Antioxidant Properties Based on Whey Derived Proteins Enriched with Hemp Seed Oil. *Antioxidants* 2020, 9, 1066; doi: 10.3390/ antiox9111066.

Submitted

Avolio E, De Lorenzo A, Olivito I, Eleonora R, Romano L, Angelone T, **De Bartolo A**, Scimeca M, Bellizzi D, D'Aquila P, Passarino G, Alò R, Facciolo RM, Bagni C, Canonaco M. Modifications of behavior and inflammatory factors in mice gut-brain axis following transplant with autistic children fecal microbiota.

Awards:

- Winner of the “VINCI 2019” Chapter II: Mobility contributions for PhD thesis in co-tutorship. Project n. C2-1563, PhD thesis in co-tutorship entitled "Metabolic effects of Selenoprotein T"

- Scholarship for a Research Project about the role of Selenoprotein T and its derived peptide, PSELT, in pathophysiological contexs. Cardio-oncology, XV Scientific Conference of the Lilli Funaro Foundation (March, 2019)

Poster communications:

XXIII Congress of the Italian Society of Cardiovascular Reaserch (Imola, 28-30 October 2021). **De Bartolo A**, Rocca C, Grande F, Rizzuti B, Pasqua T, Giordano F, Granieri MC, Occhiuzzi MA, Garofalo A, Amodio N, Cerra MC, Schneider F, Panno ML, Metz-Boutigue MH, Angelone T. Cateslytin abrogates lipopolysaccharide-induced cardiomyocyte injury by reducing inflammation and oxidative stress through toll like receptor 4 interaction.

PhD student:

Tutor:

Director of Doctorate School in *Life Sciences*:

November 15th, 2021

University of Calabria

Rende (CS), 87036

Italy

UNIVERSITÀ DELLA CALABRIA

DICHIARAZIONE PER IL DEPOSITO NELL'ARCHIVIO ISTITUZIONALE DELLE TESI DI DOTTORATO

Al Magnifico Rettore
Università della Calabria
SEDE

Io sottoscrittA ANNA DE BARDOLO

natA a COSENZA il 24/05/1992

titolo della tesi: THE SELENOPROTEIN T- MIMETIC (PSELT) EXERTS CARDIOMEMBOLIC PROTECTION IN RAT AND MOUSE MODELS OF OBESITY AND METABOLIC SYNDROME

soggetto della tesi IMPLICAZIONI CARDIOMETABOLICHE DELLA SELENOPROTEINA T

parole chiave (indicarne 5) OBESITA', STRESS OSSIDATIVO, SELENOPROTEINA T, CARDIOPROTEZIONE, PEPTIDI

denominazione della scuola/corso di dottorato:
DOTTORATO DI RICERCA IN "LIFE SCIENCES" XXXIV CICLO

dipartimento: DIPARTIMENTO DI BIOLOGIA, ECOLOGIA E SCIENZE DELLA TERRA (DiBEST)

tutor: PROF. TOMMASO ANGELONE DR. YOUSSEF ANOUAR

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- di prendere atto che la copia depositata nell'Archivio Istituzionale delle tesi di dottorato è l'unica alla quale farà riferimento l'Università della Calabria per rilasciare, a mia richiesta, la dichiarazione di conformità di eventuali copie;

- che il contenuto e l'organizzazione della tesi è opera originale da me realizzata e non compromette in alcun modo i diritti di terzi, e che pertanto l'Università della Calabria è in ogni caso esente da qualsiasi responsabilità di qualsivoglia natura, civile, amministrative o penale e sarà da me tenuta indenne da qualsiasi richiesta o rivendicazione da parte di terzi;

- che la tesi di dottorato non è il risultato di attività rientranti nella normativa sulla proprietà industriale, non è stata prodotta nell'ambito di progetti finanziati da soggetti pubblici o privati con vincoli alla divulgazione dei risultati, non è oggetto di eventuali registrazioni di tipo brevettuale o di tutela, oppure, **qualora la tesi sia oggetto di registrazione di tipo brevettuale o di tutela (barrare una delle caselle di interesse):**

  - o che il contenuto della tesi è in corso di pubblicazione (la tesi non sarà consultabile per un periodo di 12 mesi a partire dalla data di conseguimento del titolo di dottore di ricerca);

  - ø che la tesi è oggetto di registrazioni di tipo brevettuale o di tutela (la tesi non sarà consultabile per un periodo di 24 mesi a partire dalla data di conseguimento del titolo di dottore di ricerca);

Si allega una copia della presentazione del Collegio dei Docenti, firmata dal Coordinatore.

Data 27/01/2022

Firma del dottorando

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PER PRESA VISIONE
IL COORDINATORE DEL CORSO

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I dati inseriti saranno trattati unicamente per finalità istituzionali dell'Università della Calabria (Codice in materia di protezione dei dati personali – d.lgs. 30/6/2003, N. 196).