UNIVERSITÀ POLITECNICA DELLE MARCHE

SCUOLA DI DOTTORATO DI RICERCA DELLA FACOLTÀ DI MEDICINA E CHIRURGIA

Curriculum: Scienze biomediche

XXXI ciclo

NICOTINAMIDE N-METHYLTRANSFERASE INHIBITION IN ENDOTHELIUM: EFFECT ON CELL VIABILITY AND RESPIRATION

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Triennio 2015-2018

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1. INTRODUCTION

1.1 NICOTINAMIDE N-METHYLTRANSFERASE

1.1.1 Drug metabolism

Drug metabolism can be defined as the biochemical modification of drugs, occurring usually through specialized enzymatic systems that change the chemical structure of drugs to compounds that can be easily eliminated from the body. Through these biochemical processes, lipophilic chemical compounds (drugs) are converted into highly polar water-soluble derivatives which are excreted into urine or bile. Although the major site for drug metabolism is the liver, xenobiotic metabolizing enzymes may also be present in different organs such as lung, kidney and intestine.

Biotransformation of drugs can be divided into two broad categories. Phase I biotransformation reactions introduce or unmask functional groups on the drug to increase the polarity of the compound. Phase I reactions involve two groups of enzymes, such as oxidoreductase (cytochrome P450 monooxygenases, flavin monooxygenases, monoamine oxidases, aldehyde dehydrogenase, alcohol dehydrogenase) and hydrolase (amidases, esterases, epoxide hydrolase).

In phase II reactions, endogenous compounds or activated xenobiotic metabolites undergo a conjugation reaction with endogenous polar molecules resulting in highly water-soluble conjugated metabolites ready to be excreted in the urine. These conjugation reactions are catalyzed by enzymes, such as glutathione S-transferase, glucuronosyltransferase, sulfotransferase, N-acetyltransferase, and methyltransferase [1]. Among phase II reactions, methyl conjugation plays an important role in the metabolism of many drugs, xenobiotic compounds, and neurotransmitters.

Methylation of pyridine compounds is known since 1884 when it was described for the first time by Wilhelm His. The methyl donor for this reaction, as well as for the

methylation of most other drugs and xenobiotics, is the S-adenosyl-L-methionine (SAM). Human methyltransferases catalyze the reactions of S-methylation, O-methylation and N-methylation. S-methylation plays a key role in the biotransformation of drugs such as 6-mercaptopurine, D-penicillamine, and captopril, catalyzed by thiopurine methyltransferase (TPMT, E.C. 2.1.1.67), and thiol methyltransferase (TMT, EC 2.1.1.9). Catechol-O-methyltransferase (COMT, E.C. 2.1.1.6) and phenol O-methyltransferase (POMT, E.C. 2.1.1.25) are involved in the metabolism of several catecholamine neurotransmitters. N-methylation reactions are catalyzed by several N-methyltransferase in human, including histamine N-methyltransferase (HNMT, E.E. 2.1.1.8) and nicotinamide N-methyltransferase (NNMT, E.C. 2.1.1.1) [2, 3].

1.1.2 Nicotinamide homeostasis

Vitamin B3, also known as niacin or vitamin PP, owes its biological effect to two functionally related forms of such vitamin: nicotinamide and nicotinic acid.

The cell uses nicotinamide as precursor for the synthesis of both NAD^+ (Nicotinamide Adenine Dinucleotide) and NADP+ (Nicotinamide Adenine Dinucleotide Phosphate). These coenzymes play a crucial role in cell energy metabolism, taking part to ATP production through redox reactions. Moreover, both NAD+ and NADP+ play also a role in non-redox mechanisms which lead to the cleavage of the β-N-glycosidic bond and release of nicotinamide. These mechanisms include:

- histones and transcription factors deacetylation catalyzed by NAD(+)-dependent histone deacetylases (sirtuins) [4, 5],
- ADP-ribosylation of proteins catalyzed by mono-ADP-ribosyltransferase (ARTs) and poly ADP-ribose polymerases (PARPs) [6],

- production of effector molecules such as cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NaADP), that originate from CD38 molecule catalysis and are able to mobilize intracellular calcium stores [7].

Vitamin B3, present in a variety of foods, is mostly obtained from the diet and absorbed in the gastrointestinal tract by a mechanism of passive diffusion at high concentrations or by sodium ion-dependent facilitated diffusion in case of lower concentrations [8]. In addition to diet uptake, Vitamin B3 may be also synthesized using tryptophan as precursor.

Although mammals are able to synthesize nicotinamide-containing nucleotides through the kynurenine pathway in case of poor availability of niacin, this "salvage pathway" to convert tryptophan to niacin is not very efficient as it is a nine-step process and approximately 60 mg of dietary tryptophan are needed to produce only 1 mg of nicotinamide [9, 10].

The liver plays an important role in regulation of serum nicotinamide levels through the NAD+ storage. In fact, excess serum nicotinamide can be converted to NAD+ by hepatic enzymes, or, alternatively, hepatic NAD glycohydrolases can release nicotinamide in blood, which is then transported to other tissues [11].

1.1.3 Characterization of human NNMT

Nicotinamide N-methyltransferase (NNMT) is a phase II metabolizing enzyme which plays an important role in the biotransformation and detoxification of many xenobiotic compounds [3]. NNMT uses S-adenosyl-L-methionine as methyl donor to catalyze N-methylation of nicotinamide, pyridines and other structural analogs, forming positively charged pyridinium ions [12]. The products of the reaction catalyzed by NNMT are N1-

methylnicotinamide (MNA) and S-adenosyl-L-homocysteine (SAH), the latter hydrolyzed to L-homocysteine and adenosine. N1-methylnicotinamide may be excreted directly through urine or, alternatively, converted by aldehyde oxidase to N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-5-carboxamide, compounds that are excreted into urine as well (Figure 1) [13]. Although various N-methyltransferases are able to catalyze the N-methylation of aza-heterocyclic compounds, NNMT is the unique enzyme that uses nicotinamide as methyl acceptor, thus playing a key role in nicotinamide catabolism [14, 15].

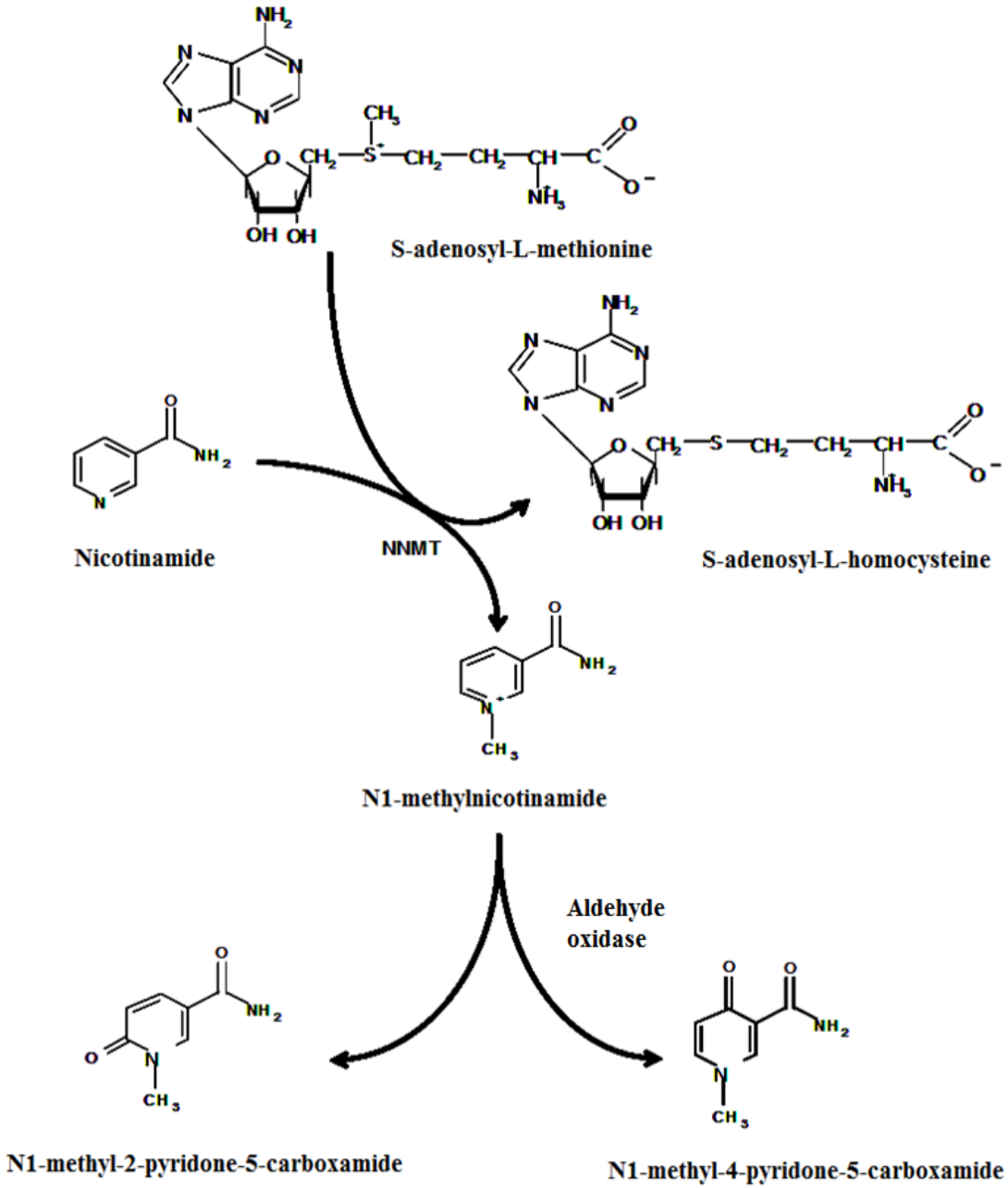


Figure 1. Nicotinamide methylation and oxidation of N1-methylnicotinamide by NNMT and aldehyde oxidase, respectively.

The characterization of human NNMT was performed for the first time in liver tissue samples and required the development of a radiochemical assay to assess the enzyme activity. This 4-steps assay includes:

- the set-up of a mix including [^{14}C-methyl]-S-adenosyl-L-methionine,
- extraction of N1-methylnicotinamide by 60% isoamyl alcohol in toluene, with 1-heptansulfonic acid as a phase modifier,
- separation and identification of N1-methylmicotinamide by reversed-phase HPLC,
- the dosage of radioactivity through a liquid scintillator.

Human hepatic NNMT exhibited a cytoplasmic distribution with a pH optimum of 7.4. Calculated Km values for nicotinamide and S-adenosyl-L-methionine were 347 µM and 1.76 µM, respectively. The mean NNMT specific activity measured in liver biopsy samples was 51,5 ± 32,5 U/mg but individual tissue samples showed poor homogeneity in terms of specific activity detected.

Activities showed a bimodal distribution with nearly 26% of the samples that were included in a subgroup with high NNMT activity, suggesting that the variations observed in NNMT activity could be one of the factors involved in individual differences in the metabolism and toxicity of pyridine compounds and implying the possibility that human NNMT activity might be regulated by a genetic polymorphism within the gene sequence [16–18].

In order to explore this hypothesis, human liver NNMT was partially purified by ion exchange followed by gel filtration chromatography. The sample obtained underwent SDS-PAGE, followed by photoaffinity labeling of NNMT that allowed the identification of a protein with a molecular mass of approximately 29 kDa. In the last step, it was performed a chemical and enzymatic proteolysis of the partially purified

NNMT and through the analysis of the amino acid sequence of generated fragments it was possible to identify the cDNA sequence. The NNMT cDNA length was 792 bp and encoded for a protein composed by 264 amino acids with a calculated molecular mass of 29.6 kDa. Finally, the NNMT cDNA was inserted into the eukaryotic expression vector p91023(B) and the final construct was subsequently used to transfect COS-1 cells. Experiments of substrate kinetic and enzyme inhibition were performed to compare human liver NNMT and transfected COS-1 cell NNMT, and the following kinetic parameters have been identified:

- Km values for nicotinamide of 0.43 mM for the native protein and 0.38 mM for the recombinant protein,
- Km values for S-adenosyl-L-methionine of 1.8 μM for the native protein and 2.2 μM for the recombinant protein,
- IC_{50} values for the inhibition of NNMT by N1-methylnicotinamide of 60 μM for the native protein and 30 μM for the recombinant protein [19].

Further analysis identified the chromosomal localization of the human NNMT gene on the chromosomal region 11q23.1, with a nucleotide sequence of approximately 16.5 kb in length, that includes 3 exons and 2 introns.

The transcription start site in the NNMT gene sequence occurs at, or close to, a nucleotide located at -108 bp upstream from the transcription initiation site, and approximately 30 nucleotides downstream from an atypical TATA box element (TCTAA) (Figure 2) [20].

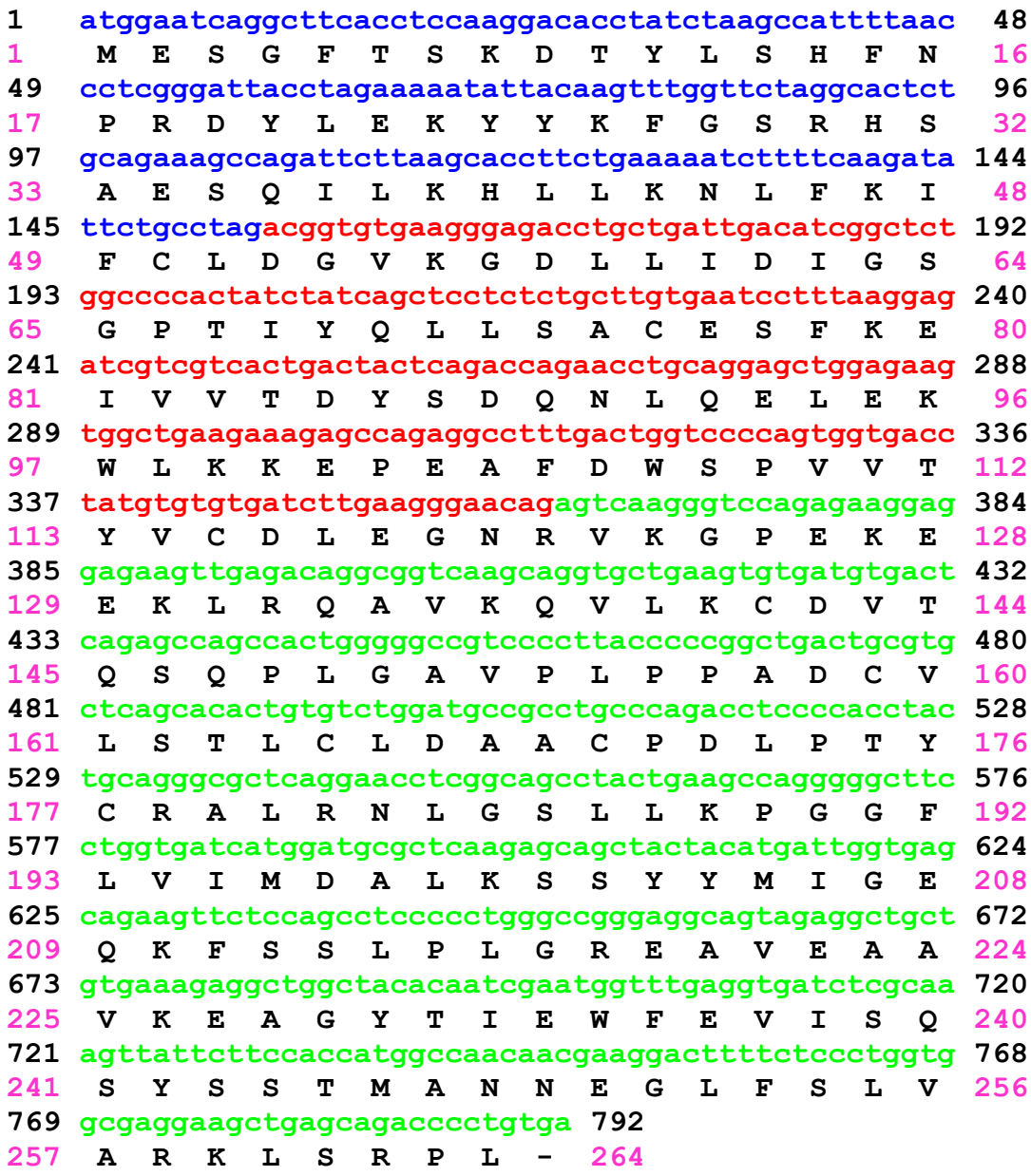


Figure 2. Human NNMT coding sequence. Exons are showed in different colours. The amino acid sequence of the encoded protein is shown in single-letter code beneath the nucleotide sequence.

Several research groups have made efforts to identify the transcription factors involved in modulating NNMT expression, demonstrating that NNMT promoter activity is regulated by molecules such as STAT3, IL-6, HNF-1β, and TGF-β1.

The expression of the NNMT gene has been reported in association with activation of signal transducer and activator of transcription 3 (STAT3) in colorectal tumor tissues. STAT3 was identified as key component of cytokine signaling pathways such as leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), activated by tyrosine phosphorylation. The NNMT promoter region (1–3 kb upstream of the gene) was cloned and fused to a luciferase reporter vector and the construct was transfected into 293 embryonic kidney and Hep-G2 hepatoma cells. Results showed that the promoter activity was stimulated by LIF and IL-6 respectively. Moreover, Stat3 inhibition in HT29 colon cancer cells with siRNA or curcumin, which inhibits Stat3 phosphorylation, resulted in a reduction of NNMT expression level [21].

The expression of the NNMT gene has been reported to be positive correlated to HNF-1β expression level in papillary thyroid cancer cell lines. After identifying a supposed HNF-1β binding site in the NNMT basal promoter region, mutations in this site abolished the capacity of HNF-1β to bind this sequence and significantly decreased the NNMT promoter activity in the HNF-1β-positive papillary thyroid cancer cell line BHP 2-7, suggesting that HNF-1β acts as a transcription activator of the NNMT promoter [22]. Moreover, both mRNA and protein NNMT expression level were found to be reduced in BHP 18-21 papillary thyroid cancer cells after treatment with the histone deacetylase inhibitor depsipeptide, and it was proven that NNMT inhibition by the depsipeptide occurs at transcription level and is mediated by the downregulation of the transcription activator HNF-1β [23].

In addition, it was also reported that TGF-β1 could modulate the NNMT expression. Indeed, it was demonstrated a downregulation of TGF-β1 and its target genes, including NNMT in human insulinomas compared to normal pancreatic islets [24]. Furthermore, a recent study reported that TGF-β1 up-regulation could be responsible for the high levels of NNMT observed in clear cell renal cell carcinoma compared to controls, strengthening the hypothesis that TGF-β1 is able to positively regulate the NNMT expression [25].

The enzyme is mostly expressed in the liver but NNMT expression, though at lower levels, was also detected in other organs and tissues such as the lung, kidney, bladder, placenta, heart, brain, and skeletal muscle [19, 20].

Analysis by X-ray diffraction, conducted on crystals obtained from the recombinant human protein purified to homogeneity, led to the resolution of the three-dimensional structure of the NNMT. Subsequent experiments of site-directed mutagenesis allowed to identify the amino acid residues mainly involved in the catalysis: tyrosine 20 (Y20) and aspartic acid 197 (D197) (Figure 3) [26].

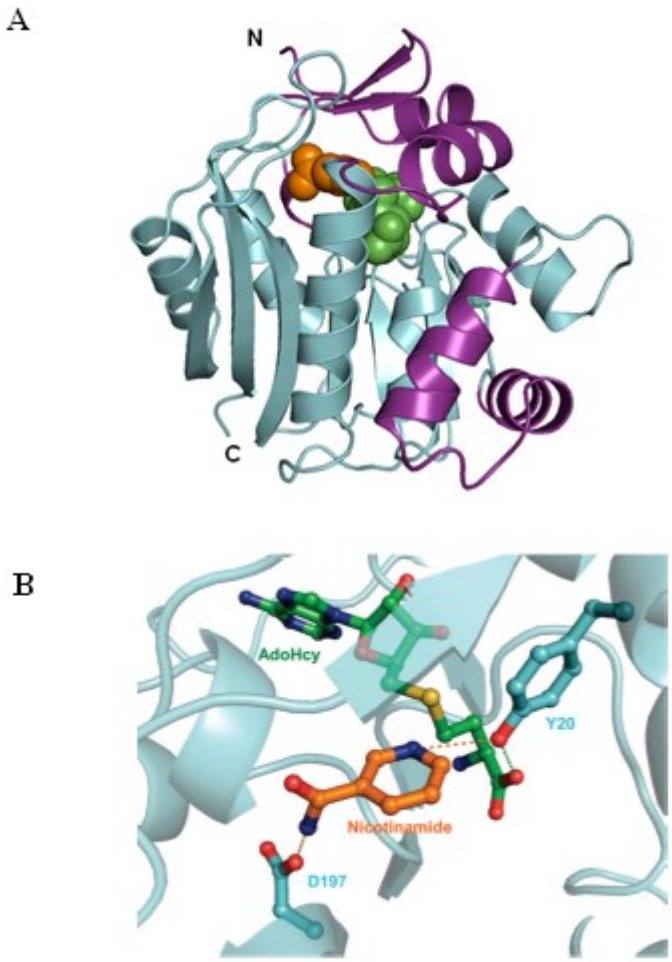


Figure 3. Crystal structure of human NNMT bound to S-adenosyl-L-homocysteine and nicotinamide (A). Y20 and D197 residues in the active site playing a critical role for NNMT catalysis (B).

1.1.4 NNMT polymorphisms

Some research groups have hypothesized that the phenotypic differences observed in nicotinamide N-methyltransferase activity could be the consequence of a genetic polymorphism within the coding regions of the gene.

In order to explore this hypothesis, it was analyzed the presence of genetic polymorphisms in the sequence of the three exons, intron 1, and 5'-flanking region of the NNMT gene in human liver biopsy samples, in which the NNMT activity was classified as low, intermediate or high level.

While no single nucleotide polymorphisms (SNPs) or insertion/deletion events were identified within either the exons or the 5'-flanking region, eight SNPs were detected within intron 1, although none of these correlated with the NNMT activity level.

Therefore, the individual variability noticed in NNMT activity could not be attributed to structural differences of the enzyme but, instead, were attributed to differences in the intracellular NNMT mRNA and protein levels [27, 28].

In a study on venous thrombosis it was performed a genome-wide linkage scan for genes affecting variation in plasma Hcy levels that revealed the presence of a genetic determinant at chromosome 11q23, corresponding to NNMT. The subsequent haplotype analyses for ten single nucleotide polymorphisms (SNPs) in the NNMT gene identified one SNP (rs694539) that resulted strongly related with homocysteine levels. This SNP is localized in the intron 1, therefore it was hypothesized that this noncoding SNP could be involved in the regulation of NNMT gene transcription [29].

Since NNMT plays a critical role in homocysteine metabolism, a case-control family study explored the relation between the NNMT polymorphism and the risk of developing congenital heart defects (CHDs), where maternal hyperhomocysteinemia is known to be risk factor. The results of this study showed that children carrying the

nicotinamide N-methyltransferase polymorphism rs694539 (AG/AA genotype) and exposed to periconception medicines and low nicotinamide intake have an eight-fold increased risk for congenital heart defects (CHDs) in [30].

Another study got an insight in the potential correlation between genetic variations of NNMT gene and spina bifida, since maternal hyperhomocysteinemia is considered an important risk factor for neural tube defects. The study involved 251 infants with spina bifida and 335 controls and, though eleven SNPs of the NNMT gene were evaluated, no correlation between infant NNMT gene variants and risk for spina bifida was found [31].

A number of studies suggested an association between abdominal aortic aneurysm (AAA) and hyperhomocysteinemia, implicating that NNMT gene variants may contribute to AAA susceptibility. 56 polymorphisms of genes involved in methionine metabolism were assessed in 423 AAA patients and 423 matched controls through a primer extension based microarray technology. Results of these studies showed that seven haplotypes, including one screened for NNMT, were significantly associated with abdominal aortic aneurysm.

A similar study was performed in a group of 501 young patients who survived ischemic stroke and 1,211 controls. However, in this case no significant correlation between the disorder and the NNMT variants was detected [32, 33].

Another work investigated whether risk of acute lymphoblastic leukemia (ALL) could be affected by polymorphisms within folate pathway genes. Analyses performed on samples collected from 245 pediatric ALL patients (cases) and 500 blood bank donors (controls) identified polymorphisms in MTHFR (677 C>T), NNMT (IVS-151 C>T), RFC1 (80G>A) genes. The NNMT IVS-151 TT variant was associated with increased susceptibility to ALL [34].

The NNMT variants rs694539, previously reported to be correlated with hyperhomocysteinemia, and the rs1941404 were significantly associated with schizophrenia (SZ) in a comprehensive association study. Moreover, NNMT mRNA levels in post-mortem frontal cortex were found to be 35% lower in SZ patients compared to control subjects suggesting that NNMT could be involved in the etiology of this disorder [35].

In another work, a case-control study within a Han Chinese female population, it was found again a correlation between the NNMT rs694539 polymorphism and schizophrenia, confirming the significant role of NNMT in the pathogenesis of this disease [36].

Recently, a number of studies examined the potential role of the NNMT rs694539 polymorphism in the development of diseases associated with high plasma homocysteine level. Other reports suggested that rs694539 NNMT polymorphism may be a genetic risk factor for, nonalcoholic steatohepatitis, epilepsy and bipolar disorders. Furthermore, individuals with the AA genotype showed an increased risk for epilepsy and nonalcoholic steatohepatitis, while GG genotype displayed protection against both diseases.

Although the biological implications of the rs694539 NNMT variant are still unclear, alteration of epigenetics, raised homocysteine levels, as well as variations of the nicotinamide levels may be one of the causes for the development of such pathologies [37–39].

1.1.5 NNMT and neoplastic diseases

Analysis of gene expression profile in glioblastoma multiforme (GBM) tissue samples revealed an upregulation of NNMT in GBMs compared to normal tissues [40]. Moreover, in a recent study experiments of NNMT silencing through shRNAs showed that NNMT plays a key role in altering biochemical and cellular functions in glioblastoma by repressing the activity of the tumor suppressor enzyme PP2A in glioblastoma [41].

In a recent study, NNMT was preferentially expressed by mesenchymal glioblastoma stem cells (GSCs). Targeting NNMT expression reduced cellular proliferation, self-renewal, and *in vivo* tumor growth of mesenchymal GSCs [42].

Furthermore, in human glioma cells cultured in the presence of interferon-γ (IFN- γ) it was detected high NNMT activity and MNA levels [43].

To give an insight in the molecular pathogenesis of thyroid cancer, the gene expression profiles of different human thyroid cancer cell lines (papillary, medullary, follicular, and anaplastic carcinomas) were analyzed through DNA microarray.

In papillary cancer cells it was found a high NNMT expression level compared to the other tested cancer cell lines, where low or no NNMT expression was detected and NNMT catalytic activity was also correlated with the mRNA expression level.

In human thyroid specimens the immunohistochemical staining for NNMT showed strong cytoplasmic reactions in follicular and papillary carcinomas, while only faint or scanty reactions were detected in follicular adenomas, colloid goiters, and normal thyroid tissues [44].

In a study performed on human breast adenocarcinoma cell line it was found that NNMT expression was detectable in adriamycin resistant MCF-7 cells but not in parental MCF-7 cells [45, 46].

In a more recent study in different human breast cancer cell lines, NNMT expression was modulated in order to assess variations in cellular viability. NNMT downregulation in Bcap-37 and MDA-MB-231 cells significantly reduced cell growth *in vitro* and tumorigenicity *in vivo*. Moreover, after NNMT silencing it was observed an increased ROS production and induced apoptosis via the mitochondria-mediated pathway. The influence of the enzyme on cell proliferation and apoptosis was then confirmed by NNMT overexpression in MCF-7 and SK-BR-3 cell lines, suggesting that the enzyme could be considered a promising molecular target in the breast cancer treatment [47].

In the field of the pancreatic cancer, it was performed an interesting study of gene expression profile from RNA isolated from pancreatic juice. It was observed an increased NNMT expression level in the pancreatic juice of patients affected by pancreatic cancer compared with controls [48].

A metabolomics analysis conducted in miR-1291-expressing and control PANC-1 pancreatic cancer cells revealed elevated concentration of MNA in miR-1291-expressing PANC-1 cells which were also found to be correlated with NNMT mRNA levels. In addition, miR-1291 stable expression in PANC-1 cells diminished cell migration, invasion and xenograft tumorigenesis compared to controls. Interestingly, an inverse relationship was uncovered between xenograft pancreatic tumor size and cancer cell NNMT mRNA levels, suggesting an important role for NNMT in miR-1291-altered PANC-1 cell metabolome and carcinogenesis, thus leading to hypothesize that NNMT may also be indicative of the extent of pancreatic cancer progression [49].

The biological function of NNMT in pancreatic cancer was also investigated in another study, showing that NNMT silencing significantly inhibited cell proliferation, and suppressed both the migration and invasion capacities of PANC-1 cells.

In addition, overexpressing NNMT improved the survival of PANC-1 cells subjected to either glucose deprivation or glycolytic inhibition, or after treatment with rapamycin, suggesting that the enzyme plays a not negligible role in pancreatic cancer cell metastatic potential and survival in condition of metabolic stress [50].

In a comparative study that included pancreatic cancer tissue samples, paracancerous tissues and chronic pancreatitis, it was found that elevated NNMT expression levels were cancer-specific. In addition, it was found a correlation between high NNMT expression level and unfavorable clinicopathological characteristics in pancreatic cancer patients, thus highlighting this parameter as an unfavorable prognostic factor of overall survival [51].

Protein expression pattern was analyzed in healthy and malignant colorectal tissues using a proteomics approach, with the purpose to identify cancer-related proteins. Detected NNMT levels were remarkably higher in colorectal cancer compared with those in adjacent normal tissue samples. Furthermore, in serum from patients with colorectal cancer it was detected higher NNMT level compared to controls, proposing the enzyme as a novel biomarker for the detection of colorectal cancer [52].

A recent work gave an insight into the biological function of NNMT in human colorectal cancer cell lines. Overexpressing NNMT in SW480 cells, which do not have a constitutive NNMT expression, led to an increase in tumorigenicity capacity both *in vitro* and *in vivo*, inhibited apoptosis and promoted cell cycle progression. Furthermore, it was observed an increased intracellular ATP level and a decrease in ROS production in case of NNMT overexpression.

It was demonstrated that the observed cellular effects of NNMT were due to the increased MNA levels, thus suggesting that the enzyme could be involved in energy balance and ROS production [53].

The impact of the NNMT on chemotherapy sensitivity was also investigated in colorectal cancer cells, by focusing on 5-fluorouracil (5-FU). In SW480 cells the NNMT overexpression resulted in a decrease of the 5-FU-induced apoptosis and reduction of the apoptosis-related proteins levels, such as caspase-3, caspase-8, and caspase-9.

Moreover, it was demonstrated that the decreased apoptosis was a consequence of NNMT activity that inhibited the activation of the ASK1-p38 MAPK pathway, thus enhancing colorectal cancer cells resistance to 5-FU.

These data suggested that NNMT is involved in the resistance to 5-FU, and therefore it may represent a promising therapeutic target for the colorectal cancer treatment [54].

In a comparative proteome analysis performed in gastric cancer samples, it was found a remarkable difference in NNMT protein expression between stomach adenocarcinoma compared with matched normal tissues, with a higher expression for cancer tissues [55].

Subsequent analysis, with the aim to identify novel biomarkers, confirmed elevated NNMT expression in gastric cancer tissue [56].

In addition, in gastric cancer tissue samples subjected to two-dimensional gel electrophoresis and subsequently to western blot, it was detected the presence of multiple spots of NNMT whereas only one spot was detected in normal and gastric ulcer tissues, suggesting that NNMT may receive post-translational modifications in a cancer-specific manner [57].

In a recent study, it was examined the biological and prognostic function of the NNMT in gastric carcinoma. Both NNMT mRNA and protein levels resulted upregulated in gastric carcinoma tissues compared with normal adjacent tissues and experiments of NNMT gene silencing in MGC803 and BGC823 gastric cell lines showed a decrease in cell proliferation, invasion and migration *in vitro* and *in vivo*.

Furthermore, NNMT expression resulted correlated with tumor size, lymph node infiltration and distant metastasis, TNM stage, and overall survival, suggesting that the enzyme could be a novel prognostic factor in the gastric carcinoma [58].

Overexpression of NNMT in human gastric cancer cell line BGC-823 significantly induced the expression of TGF-β1, therefore activating TGF-β1/Smad signaling, which in turn promotes epithelial-mesenchimal transition (EMT), suggesting an important role for NNMT in enhancing invasive ability of cancer cells [59].

In a work with the purpose to investigate the involvement of drug-metabolizing enzymes in clear cell renal cell carcinoma (ccRCC), experiments of gene expression profiles analysis detected increased NNMT levels in tumor tissue compared with normal renal tissue. Moreover, the tumor size resulted inversely correlated with NNMT mRNA levels, suggesting a potential role for the enzyme in the tumor growth [60, 61].

The upregulation of NNMT also at protein level in renal cell carcinoma was then confirmed by immunohistochemical analysis and high NNMT level was significantly correlated with a poor prognosis [62].

High NNMT levels were also detected in the plasma of RCC patients and in the tumor tissue interstitial fluid compared to normal controls, raising the prospective that NNMT may be used as a diagnostic biomarker for renal cancer [63–65].

In a recent study, a research group got an insight into the potential role of NNMT in the cellular invasion of ccRCC cells, showing that NNMT gene silencing suppressed invasive capacity *in vitro* and inhibited tumor growth and metastasis *in vivo* [66].

Immunohistochemical analysis performed in benign and malignant prostate tissue samples revealed a significant high NNMT level in prostate cancer compared with benign prostate hyperplasia.

Furthermore, it was demonstrated an inverse correlation between NNMT expression and Gleason score, whereas high NNMT expression was associated with prolonged progression-free survival and overall survival in patients with advanced prostate cancer. These observations arose the possibility that NNMT could become a prognostic biomarker for prostate cancer [67].

NNMT expression was measured in the peripheral blood of patients with prostate cancer and it was found higher compared to healthy control subjects. In addition, the overexpression of NNMT enhanced PC-3 cell viability, invasion and migration capacity through the mediation of SIRT1, indicating that NNMT is an important regulator of SIRT1 expression in PC-3 cells and can be a potential therapeutic target for prostate cancer [68].

An investigation of the expression profile of stress-related and DNA repair genes in a radioresistant bladder carcinoma cell line (MGH-U1) and its radiosensitive subclone (S40b) revealed high NNMT mRNA levels in MGH-U1 compared to S40b, suggesting a possible role of the enzyme in predicting radiation response, thus proposing its potential use as target for cancer therapy [69].

Subsequent studies were conducted in order to elucidate the source responsible for the radiation resistance in cancer stem cells (CSCs). The overexpression of NNMT in tumorigenic mesenchymal CSC clones strongly enhanced their radiation resistance, implying an association between high NNMT level and the observed radiation response. Increasing of the NNMT activity may potentially reduce intracellular nicotinamide concentration, with the consequence to reduce PARP inhibition, normally operated by nicotinamide, and thus resulting in DNA damage repair.

In addition, NAMPT, the enzyme that allows the conversion of nicotinamide into NAD+, was upregulated in the CSC clones after irradiation. Therefore, high NAMPT

activity can increase the intracellular NAD+ level, the essential substrate for DNA repair activity-PARP mediated [70].

In a recent study, in CSC-enriched population obtained from Hep-2 cell line both mRNA expression and catalytic activity of NNMT were evaluated. Results obtained indicated that NNMT levels were higher in CSC-enriched populations compared to the parental counterpart. Considering that CSCs play a crucial role in early tumor formation and maintenance, the previous observations suggested that NNMT could be involved in cancer cell metabolism [71].

In order to discover the key molecules involved in cellular invasion, gene expression profiles were examinated in human bladder cancer cell lines and it was found a positive correlation between NNMT expression and both cancer cell migration and tumor stage. Moreover, NNMT silencing in bladder cancer cells resulted in a significant reduction of cell proliferation and migration, suggesting a possible role of the enzyme in tumor progression [72].

In a recent study, in bladder urothelial carcinoma (UC) it was detected higher NNMT expression compared with adjacent normal looking tissue, and also a significant increase in NNMT enzymatic activity was measured in all cancerous specimens. Furthermore, NNMT expression was also assessed in exfoliated urinary cells collected from urine samples of patients with bladder UC and healthy controls.

Data revealed an increased NNMT expression, for both mRNA and protein, in bladder cancer compared to control specimens, proposing the enzyme as a novel biomarker for early and non-invasive detection of bladder cancer [73].

A recent study analyzed NNMT expression in urine samples from bladder cancer patients using real-time polymerase chain reaction (PCR). Receiver operating characteristic (ROC) analysis and area under the curve (AUC) values showed an

excellent diagnostic accuracy of this novel urine-based NNMT test, indicating that NNMT could be used as biomarker to support the early and non-invasive diagnosis of bladder cancer [74]. In addition, further analysis demonstrated a NNMT upregulation in muscle-invasive bladder cancer compared to non-invasive samples [75].

With the purpose to identify a potential biomarker of lung cancer, an ELISA system was set up and revealed a significant increase of serum NNMT level in non-small cell lung cancer (NSCLC) patients compared with healthy donors and patients with chronic obstructive pulmonary disease (COPD). Furthermore, statistical analysis allowed to determine that sensitivity of NSCLC detection was higher when NNMT was used in combination with the carcinoembryonic antigen (CEA), the well-known lung cancer tumor marker [76].

Another work evaluated NNMT expression levels in tumor, tumor-adjacent, and contiguous tissue samples from patients with NSCLC by different approaches such as Real-Time PCR, western blot, catalytic activity assay, and immunohistochemistry. Results showed that both NNMT mRNA and protein levels were considerably higher in tumor compared with both tumor-adjacent and surrounding tissues.

In addition, in NSCLC it was detected an increased activity of NNMT compared to those measured in both tumor-adjacent and surrounding tissue, indicating that NNMT could be taken in consideration as a novel molecular marker for NSCLC [77, 78].

In a recent study, it was demonstrated a negative correlation between the expression levels of NNMT and miR-449a in EGFR-TKI-resistant NSCLC cells. Moreover, knockdown of NNMT induced the expression of miR-449a in drug-resistant NSCLC cells and suppressed p-Akt and tumorigenesis. These data suggested that targeting NNMT might be a novel therapeutic strategy in EGFR-TKI-resistant NSCLC patients [79].

In a research on hepatocellular carcinoma (HCC) specimens, it was explored NNMT expression and its clinicopathologic relevance. NNMT mRNA level was found to be significantly reduced in HCCs compared to non-cancerous surrounding tissues. Furthermore, it was found an association between the enzyme expression and the tumor stage, whereas an inverse correlation was observed between NNMT mRNA levels and overall survival, indicating that the enzyme could be taken in consideration as a prognostic factor in hepatocellular carcinoma [80]. In a human HCC cell line, the overexpression of NNMT led to a significant increase in cellular invasion and adhesion [81].

The function of NNMT was also investigated in oral squamous cell carcinoma (OSCC). A significant augmented expression of NNMT was proved in tumor tissues compared with normal oral mucosa. In addition, NNMT mRNA overexpression in oral cancer was inversely correlated with pT, pathological staging, and lymph node metastasis [82].

Subsequent immunohistochemical analysis showed a significant inverse correlation between cytosolic NNMT protein expression and the histological grading of the tumor, proposing NNMT as a potential prognostic marker for OSCC [83].

In a recent study, it was demonstrated a higher NNMT activity level in OSCC than in surrounding normal oral mucosa. Furthermore, in saliva of patients with OSCC it was detected high NNMT expression through western blot analysis, therefore suggesting that NNMT may represent a potential biomarker for early and non-invasive diagnosis of oral cancer [84].

In a study devoted to explore the role of NNMT in cancer cell metabolism, RNA interference-mediated silencing of the enzyme was performed in KB and PE/CA-PJ15 cell lines and the influence on cell proliferation was then evaluated. NNMT

downregulation significantly reduced cell growth *in vitro* and tumorigenicity *in vivo*, suggesting that the enzyme may have a role in tumorigenesis [85, 86].

To further explore the role of the enzyme in oral cancer cell metabolism NNMT was overexpressed in HSC-2 cells and it was investigated the role of NNMT on apoptosis and cell proliferation. Data obtained showed that upregulation significantly increased cell growth *in vitro* and a positive correlation between NNMT and survivin ΔEx3 isoform expression levels was found both in HSC-2 cells and in OSCC tissue samples, confirming the possible involvement of NNMT in the proliferation and tumorigenic capacity of OSCC cells [87].

High NNMT levels were also detected in nasopharyngeal cancer tissues and were correlated with advanced tumor stage and reduced survival, suggesting a potential role of the enzyme as a prognostic factor [88].

DNA microarray study showed that NNMT is overexpressed in adenoid cystic carcinoma lymph node metastatic cells (ACCS-LN) and it was hypothesized that ACCS-LN cells acquire cancer stem cell features involving the up-regulation of NNMT [89].

In order to explore the role of NNMT in melanoma, immunohistochemical analyses were performed in melanomas and nevi samples. Data obtained showed significantly higher NNMT expression in melanoma compared to nevi. Moreover, a significant inverse relationship was found between enzyme levels and Breslow thickness, Clark level, the presence/number of mitoses, and ulceration, suggesting that NNMT could represent a molecular biomarker in melanoma, both for diagnosis and prognosis [90].

Recently, a detailed metabolomic analysis revealed that elevated NNMT activity levels can strongly influence the methylation potential of cancer cells due to a consumption of methyl units from S-adenosyl-L-methionine.

Therefore, epigenetic state of NNMT-expressing cancer cells is altered, characterized by a general hypomethylation of histones and cancer-related proteins, as well as enhanced expression of protumorigenic factors [91, 92].

Another recent study reported that NNMT plays an important role in the pluripotency state of human embryonic stem cells (hESCs), regulating the epigenetic landscape during naïve to primed transition [93].

Serous ovarian cancer cells resistant to glucose starvation demonstrated increased metabolic plasticity that was dependent on NNMT expression. It was also found that NNMT was required for other ZEB1-induced phenotypes, such as those charactezized by increased migration capacity. Furthermore, NNMT protein levels were also increased in metastatic and recurrent tumors compared to matched primary carcinomas, whereas normal ovary and fallopian tube tissue had no detectable NNMT expression. These data suggested that the enzyme can be an important target for fighting against acquired drug resistance and recurrence in high-grade serous ovarian cancer [94].

1.1.6 NNMT and Parkinson's disease

Although N-methylation is an important step of the pathway for detoxification of many xenobiotic molecules, in some cases this biotransformation can also induce the opposite effect enhancing the toxicity of some substrates. In the light of these observations, it is reasonable to hypothesize that high levels of NNMT activity in brain may trigger a overproduction of neurotoxic methylpyridinium ions, representing a factor in the etiology of Parkinson disease (PD).

In order to investigate the involvement of NNMT in the occurrence of PD, it was analyzed the regional expression of the enzyme in healthy human brain tissue and the

results showed that NNMT expression is exclusively confined to neurons with regional differences in expression levels. In particular, elevated NNMT mRNA levels were detected in the spinal cord, medulla, and temporal lobe, while low expression levels were observed in the cerebellum, subthalamic and caudate nucleus. In addition, both NNMT protein and enzyme activity were detected in the spinal cord and temporal lobe.

It was measured the NNMT expression comparing PD and non-PD control cerebella and caudate nucleus, and results showed a significant increase in the brains of dead patients affected by PD.

In addition, it was demonstrated an inverse correlation between the level of patient's NNMT expression and the duration of the disease, suggesting a causative effect in the pathogenesis of the disease [95, 96].

This hypothesis was supported also by other studies that reported the neurotoxic effect of MNA, which triggers the disruption of complex I subunits of the mitochondrial chain via the induction of free radicals [97–100].

In a recent study it was used the human neuroblastoma-derived cell line SH-SY5Y that, lacking of endogenous NNMT, can be considered an ideal research model to explore the effects of NNMT expression on cell viability and metabolism.

An experiment of stable NNMT expression significantly diminished cell death in SH-SY5Y cells, effect that was associated with enhanced Complex I activity and ATP production, therefore indicating cytoprotection exerted by the enzyme.

The same results were achieved incubating SH-SY5Y cells with MNA, suggesting that this molecule mediates the cellular effects of NNMT. Furthermore, both NNMT expression and MNA exposure demonstrated to have a protective effect on SH-SY5Y cells from the toxicity of the Complex I inhibitors MPP+ (1-methyl-4-phenylpyridinium ion) and rotenone. Taken together, these observations led to the hypothesis that NNMT

overexpression detected in PD patients could represent a stress response of the cell to the underlying pathogenic process [101].

It was demonstrated also that NNMT confers protection against mitochondrial toxicity induced by potassium cyanide (KCN), 2,4-dinitrophenol, and 6-hydroxydopamine *in vitro*, and it was proved that its neuroprotective effects are not mediated exclusively through increased MNA production, but through multiple mechanisms [102].

In another work conducted in SH-SY5Y and N27 rat mesencephalic dopaminergic cell lines it was demonstrated that the enzyme plays a role in neuron morphology and differentiation, revealing that NNMT expression increases neurite branching and also increases the production of functional synapses via the activation of the Akt-EFNB2 signaling pathway [103].

To investigate the molecular mechanisms responsible for the observed cytoprotective effects, it was examined the possibility whether sirtuins could mediates the effect of NNMT upon complex I activity. Sirt-3 silencing in NNMT-expressing SH-SY5Y cells verified the central role that this NAD-dependent protein deacetylase has in mediating the NNMT-induced complex I activity and ATP production [104].

Considering the elevated levels of both N-methylated β-carbolines (BCs) and NNMT detected in PD, in a recent work it was explored whether the enzyme could display N-methyltransferase activity towards BCs. Interestingly, it was found that recombinant NNMT was able to N-methylate the BC norharman (NH) to 2-N-methylnorharman (meNH). Subsequent analyses demonstrated that meNH displayed lower toxicity than its precursor NH, and it revealed a capacity to increase cell viability and intracellular ATP concentration in NNMT-expressing SH-SY5Y cells.

Altogether, these observations indicated an involvement of the enzyme in the detoxification pathway for BCs and that NNMT overexpression may represent a cytoprotective reaction to the pathogenesis of PD [105].

1.1.7 NNMT and non-neoplastic diseases

Although the liver represent the organ with the highest NNMT expression, the function of the enzyme in hepatocytes metabolism is still unclear.

A recent study conducted in mice showed that hepatic NNMT levels regulate glucose, lipid and cholesterol metabolism through the stabilization of SIRT1 protein, and that the effect of NNMT upon metabolism are mediated by its reaction product N1-methylnicotinamide [106].

High MNA serum levels were detected in patients affected by liver cirrhosis compared to control subjects, though this condition does not impair the efficiency of nicotinamide methylation [107].

It was detected an upregulation of NNMT gene expression in human renal allograft biopsies with histologic evidence of acute cellular rejection [108]. Moreover, in endometrial biopsies obtained from patients who became pregnant after intracytoplasmic sperm injection (ICSI) cycles, it was found NNMT overexpression, indicating a potential participation of the enzyme in the embryo implantation process [109, 110].

High NNMT expression was also detected in endometrial stromal cells in response to cytokines produced by macrophages. Thus, the increased NNMT expression could be associated with the migration and invasion of endometrial cells that takes place during the process of endometriosis [111].

NNMT SNPs were have been significantly associated with some related non-communicable chronic diseases. While SNP rs694539 was significantly associated with hyperlipidemia, SNP rs10891644 was found to be correlated with obesity [112, 113].

Analysis conducted in murine 3T3-L1 mouse embryo fibroblasts, which underwent adipogenic differentiation, as well as in human and murine adipose tissue samples showed an augmented NNMT activity level and homocysteine release.

Thus, increased NNMT activity could induce high plasma homocysteine levels, which is considered a risk factor for cardiovascular disease [114].

In numerous studies NNMT expression was found to be correlated with chronic obstructive pulmonary disease (COPD). Elevated NNMT mRNA expression was detected in quadriceps muscles of patients with COPD [115].

Furthermore, NNMT overexpression in myoblasts significantly enhanced cell proliferation and migration, as well as reduced protein oxidation and H_2O_2-induced cell death. Therefore, NNMT upregulation observed in the skeletal muscles of patients affected by COPD could improve myogenesis and defend against oxidative stress [116].

Elevated NNMT levels were also detected in lung tissues of patients with COPD, suggesting a potential involvement of the enzyme in COPD severity [117].

A recent study underlined the protective role of NNMT against mitochondrial ROS generation in the pathogenesis of proximal tubular cell (PTC) damage and consequent renal dysfunction, in patients with refractory proteinuria. In detail, MNA was demonstrated to reduce lipotoxicity-induced oxidative stress and cell damage in kidney proximal tubular cells, suggesting that supplementation of MNA may have potential as a new therapy in patients with refractory proteinuria. [118].

Conversely, increased NNMT activity levels were detected in mice treated with phenobarbital, a compound involved in ROS generation via cytochrome P450 enzymes

stimulation. The enhanced NNMT activity was correlated with the depletion of pyridine nucleotides, resulting in the attenuation of the NADPH-dependent antioxidant enzymes. Therefore, NNMT overexpression might reduce protective systems against oxidative stress [119].

In a recent work it was found that NNMT expression is increased in white adipose tissue (WAT) and liver of obese and diabetic mice. Furthermore, NNMT knockdown through antisense oligonucleotides protected mice from diet-induced obesity by augmenting cellular energy expenditure [120].

A subsequent study investigated WAT NNMT expression level in patients affected by type 2 diabetes and insulin-resistant individuals. It was detected higher WAT NNMT expression in human insulin resistance and type 2 diabetes patients compared with controls. In addition, it was found a positive correlation between the NNMT expression and the degree of insulin resistance [121].

The role of NNMT was also explored in the metabolic syndrome, and the enzyme expression was measured in the adipose tissue of Wistar Ottawa Karlsburg W (WOKW) rats, which are considered an animal model for metabolic syndrome, and Dark Agouti (DA) rats, as control. Increased NNMT mRNA, protein, and activity levels were detected in adipose tissue of WOKW rats, reinforcing the hypothesis that this enzyme could play an important role in the pathogenesis of this disorder [122].

In a recent study, it was shown that NNMT overexpression in mice fed by a NA-supplemented high fat diet (HFD) promoted hepatic steatosis and fibrosis. Since NNMT is a potent regulator of NAD+ metabolism, it was suggested that inhibiting the NNMT function could be a novel strategy to treat fatty liver and fibrosis [123].

A recent study conducted on 265 Chinese patients measured serum MNA by liquid chromatography-mass spectrometry. Results demonstrated a significant negative

association of serum MNA with left ventricular ejection fraction and preload recruitable stroke work [124].

Increased NNMT expression, at both mRNA and serum protein level, was found in patients with peripheral occlusive arterial disease compared to healthy subjects, suggesting that the enzyme could be a novel biomarker for this condition [125].

Although for a long time MNA was considered to be an inactive endogenous metabolite of nicotinamide, recent studies demonstrated that it exerts biological functions.

Topical therapy with MNA showed beneficial effects in patients with inflammatory skin diseases, including contact dermatitis and *acne vulgaris* [126]. In addition, it was reported that the anti-inflammatory effects of MNA could be correlated with its ability to reduce adherence of pro-inflammatory cells and molecules to the vascular endothelium [127].

MNA also displayed anti-thrombotic activity in rats with arterial thrombosis. While *in vitro* MNA did not influence platelet aggregation, thus excluding a direct antiplatelet action, *in vivo* it showed an anti-thrombotic effect through a mechanism involving cyclooxygenase-2 (COX-2) and prostacyclin (PGI_2).

These observations supported the hypothesis that endogenous MNA may regulate thrombosis as well as inflammatory processes characterizing cardiovascular diseases and thus biological activity of MNA observed *in vivo* may have potentially important physiological, biochemical as well as therapeutic implications [128].

According to the results obtained in the previous studies, it was investigated whether MNA could have a role in the progression of atherosclerosis since the formation and progression of atherosclerotic plaques are driven by vascular inflammation and thrombosis, and both these processes have been associated with MNA. In this study conducted in mice with atherosclerosis, the progression of the disease was associated

with increased hepatic NNMT activity and MNA plasma levels. Thus, since MNA displayed anti-thrombotic and anti-inflammatory properties, high NNMT levels could play an important compensatory role in atherosclerosis [129].

Subsequent analysis characterized in more detailed manner the response of the NNMT-MNA pathway in relation to the development of hepatitis. Data obtained revealed a significant increase of hepatic NNMT activity and MNA plasma levels during the progression of concanavalinA (ConA)-induced hepatitis in mice, suggesting a potential hepatoprotective effect of MNA through a PGI_2-dependent mechanism [130].

During endurance exercise in mice NNMT- MNA pathway was shown to be activated. Moreover, results obtained demonstrated that exercise-induced activation of NNMT in the liver involves IL-6, whereas variations in MNA concentration detected in plasma were partially IL-6-independent. Therefore, IL-6 is able to regulate hepatic NNMT activity but also other tissues may determine MNA level in plasma [131].

Changes in NNMT-MNA pathway were also examined in pulmonary arterial hypertension (PAH), a disease associated with inflammatory response. NNMT activity increased progressively in liver and lung in relation to PAH progression, and NNMT response was associated with elevated plasma MNA level. Given the vasoprotective activity exerted by MNA, the activation of the NNMT-MNA pathway may play a compensatory role in PAH [132].

1.2 AIM OF THE STUDY

An impairment of the vascular endothelium plays a role, either as a primary cause or as a result of organ damage, in most, if not all, human diseases [133].

Endothelial dysfunction in diabetes leads to macro- and microangiopathies of the cardiovascular system, leading to myocardial infarction, nephropathy, or diabetic retinopathy. Despite intensive research, biochemical and molecular mechanisms underlying endothelial dysfunction remain not fully explained. Potential factors affecting normal endothelial functions can be NNMT and MNA. Indeed, exogenously administered MNA exhibits anticoagulant [128] anti-inflammatory [130] and vascular protecting properties [134]. However, the function of endogenous MNA synthesised in endothelial cells has remained unknown, so far.

In this light, the major aim of this work was to explore the role of NNMT in endothelial cells subjected to different types (models) of stressors, including oxidative, genotoxic, and metabolic stress condition.

First, experiments were performed with the use of well-defined line of endothelial cells EA.hy926 in which the NNMT expression was silenced. For EA.hy926 cell line stable transfection, a set of pLKO.1 vectors containing stem-loop cassettes encoding short hairpin RNA (shRNA) targeted to human NNMT (pLKO.1-330, 1-448, 1-164, 1-711) was used. The efficiency of gene silencing was detected by Real-Time PCR and Western Blot analysis, while MNA production in each clone was detected through LC/MS/MS. Silenced lines were subjected to several types of stressors (Menadione, $ONOO^-$, H_2O_2, UV-C radiation). Cell viability was assessed the through MTS test and cell counting through fluorescence microscope. In addition, the percentage of apoptotic

and necrotic cells was analyzed through cytometric Annexin V/PI assay. Subsequently, the glycolytic function and the mitochondrial respiration were evaluated through the Seahorse technology.

In the second part of the work, results obtained were confirmed by using Human Aortic Endothelial Cells (HAEC) primary line, in which the MNA production was repressed by novel NNMT inhibitors.

2. MATERIAL AND METHODS

2.1 NNMT SILENCING IN EAhy.926 ENDOTHELIAL CELL LINE

2.1.1 Cell lines and reagents

The human endothelial cell line EAhy.926, purchased from the America type Culture Collection (ATCC, Rockville, MD, USA), was cultured in DMEM High glucose medium (4.5 g/l), supplemented with 10% fetal bovine serum (FBS), glutamine 2 mM and gentamicin 50 μg/ml, at 37 °C in a humidified 5% CO_2 incubator.

2.1.2 EAhy.926 electroporation

Cells grown to 80–90% confluence were removed from the T25 flask using 0.025% trypsin/EDTA (Gibco) at room temperature. 2 x 10^6 cells were resuspended in Optimem (Gibco) + 10% FBS. 20 μg of shRNA plasmids against NNMT (pLKO.1-164, 1-330, 1-448 and 1-711) or of empty pLKO.1 puro (pLKO.1 ∅) vector was mixed with the cell suspension to a final volume of 400 μl and incubated for 10 min at 4 °C. Control EAhy.926 cells (mock) were treated with transfection reagents only.

The cell suspension was placed in a 4 mm-gap electroporation cuvette (BTX). Cells were electroporated at capacitances of 900 μF in combination with 200 V.

The electroporated cells were left in the cuvette for 10 min at room temperature and then transferred in T75 flasks with complete growth medium. Forty-eight hours after transfection, stably NNMT downregulating clones were selected by maintaining cells in complete medium containing puromycin (0.4 μg/ml), with medium changes every 48 hours. All subsequent experiments were performed on selected cells growing in complete medium containing puromycin.

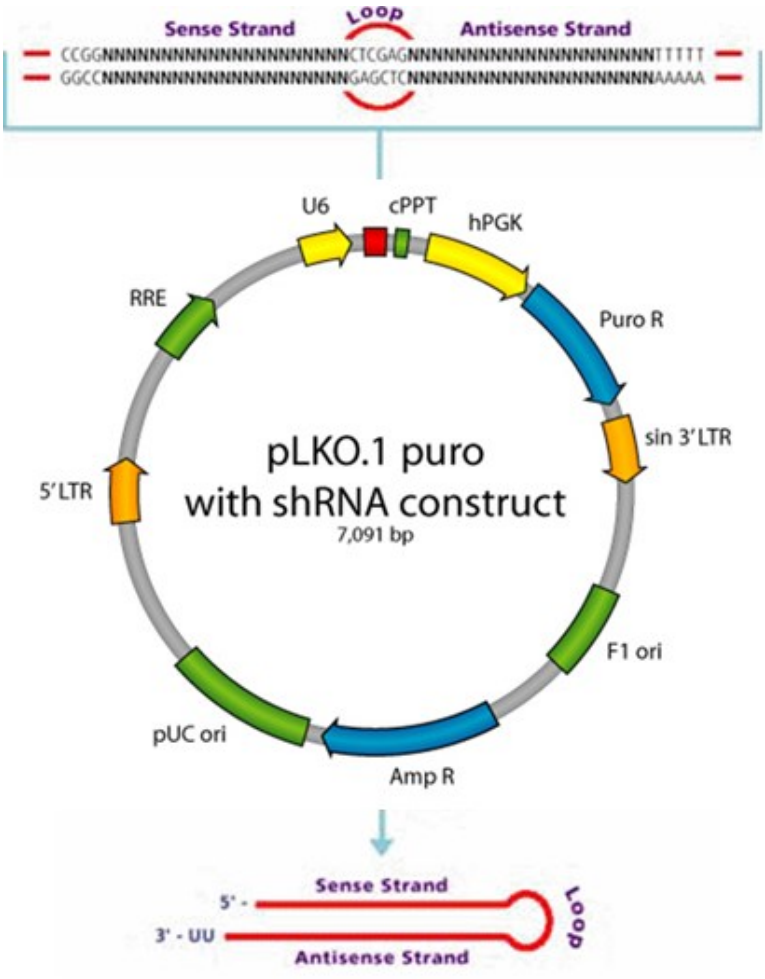


Figure 4. Plasmid pLKO.1-puro vector. The expression of shRNA encoded by the plasmid are regulated by the human U6 promoter. The vector includes a puromycin resistance gene (PuroR) for selection in mammalian cells.

2.2 EFFICIENCY OF NNMT SILENCING

2.2.1 Total RNA extraction and cDNA synthesis

Cell pellets (1 x 10^6 cells) were homogenized in lysis buffer, and total RNA was isolated through the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol.

The quantity and quality of RNA were assessed spectrophotometrically at 260 nm, 230 nm and 280 nm, and confirmed by electrophoresis. Total RNA (2 μg) was reverse transcribed in a total volume of 25 μl for 60 minutes at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), using random primers. cDNA samples were used to perform subsequent Real-Time PCR analysis.

2.2.2 Real-Time PCR

To evaluate NNMT mRNA expression quantitatively, a Real-Time PCR assay was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA, generated as described above, was used as template. To avoid false-positive results caused by amplification of contaminating genomic DNA in the cDNA preparation, all primers were selected to flank an intron, and PCR efficiency was tested for both primer pairs and found to be close to 1. The primers used were 5′-GAA TCA GGC TTC ACC TCC AA-3′ (forward) and 5′-TCA CAC CGT CTA GGC AGA AT-3′ (reverse) for NNMT, and 5′-TCC TTC CTG GGC ATG GAG T-3′ and 5′-AGC ACT GTG TTG GCG TAC AG-3′ for β-actin.

Both genes were run in duplicate for 40 cycles at 94 °C for 30 seconds and 58°C for 30 seconds, using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA,

USA). All samples were tested in triplicate using β-actin gene for data normalization in order to correct for variations in RNA quality and quantity. Direct detection of PCR products was monitored by measuring the fluorescence produced by EvaGreen dye binding to double strand DNA after every cycle. These measurements were then plotted against cycle numbers. The parameter threshold cycle (Ct) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Following NNMT gene silencing in EAhy.926 cells, fold changes in relative gene expression were calculated by $2^{-\Delta(\Delta Ct)}$ [135], where ΔCt = Ct (NNMT)- Ct (β-actin) and Δ(ΔCt) = ΔCt (cells transfected with plasmid vectors) - ΔCt (mock).

2.2.3 Western blot analysis

Western Blot experiments were performed to evaluate NNMT protein expression level. Cell pellets (2 x 10^6 cells) were suspended in 200 µl lysis buffer (phosphate buffered saline containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride and 2 µg/ml aprotinin) and homogenized by passing 3-5 times through a 30 gauge needle attached to a 1 ml syringe. After centrifugation at 16000 x g for 10 minutes at 4 °C, the supernatant containing the protein extract was collected. Samples containing 50 µg protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli method [136], using a running gel of 15% polyacrylamide. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 250 mA for 30 minutes, using a wet transfer method. PVDF membranes were blocked overnight at 4 °C in 1X phosphate buffered saline (PBS) solution containing 5% nonfat dry milk, and 0.05% tween-20. After washing three times with 1X PBS containing 0.05% tween-20, the membranes were incubated

with rabbit polyclonal antibody against NNMT (Sigma-Aldrich, St. Louis, MO, USA) (1:1000 dilution) for 1 hour, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) (1:2000 dilution) for 1 hour. NNMT protein was visualized using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal of NNMT protein detected in blots was acquired using ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.4 Protein assay

Protein concentration was measured by the Bradford method, using bovine serum albumin as the standard [137].

2.2.5 MNA measurements

Cells were seeded in 6-well plates (1.5 x 10^5 cells/well) the day before the experiment. After 24 hours, medium was removed and cells were washed twice by PBS 1X. Cells were then incubated with 500 µl Hank's Balanced Salt Solution (HBSS) buffer for 4h at 37 °C. HBSS buffer was then replaced with 500 µl of fresh one with nicotinamide 100 µM. At timepoints 0, 30, 60 min (P711) or only 60 min (P164, P330, P448) cells were detached using 0.025% trypsin/EDTA (Gibco) at 37 °C for 5 min and centrifugated at 500 g for 5 min. Intracellular MNA concentration was analyzed by LC/MS/MS. For the *ex vivo* assay, 20 mg aliquots of mice liver were washed twice by PBS 1X and then incubated with 500 µl HBSS buffer and nicotinamide 100 µM at 37 °C. At timepoints 0, 30, 60, 120, 180 min, 50 µl of extracellular fluid was collected and in each sample MNA concentration was analyzed by LC/MS/MS.

2.3 NNMT INHIBITION IN HAEC CELL LINE

2.3.1 Cell line and reagents

The human aortic endothelial cell line (HAEC) was purchased from Lonza (Basel, Switzerland). The cells were cultured in 2% FBS endothelial growth medium (EGM)-2 Bullet Kit (Lonza), according to the manufacturer's instructions, with the addition of 50 μg/ml of gentamicin. Cells were grown at 37°C in a humidified 5% CO_2 incubator. All experiments were performed using HAECs between the 2nd and the 5th passage.

2.3.2 Chemicals

To suppress NNMT activity in HAECs two novel NNMT inhibitors were used: 5-Amino-1-methylquinoline and 6-Methoxynicotinamide N-methylated (Figure 5). NNMT inhibitors were synthesized by Prof. J. Młynarski (The Institute of Organic Chemistry of the Polish Academy of Sciences, Warsaw, Poland) through published synthesis schemes [138, 139].

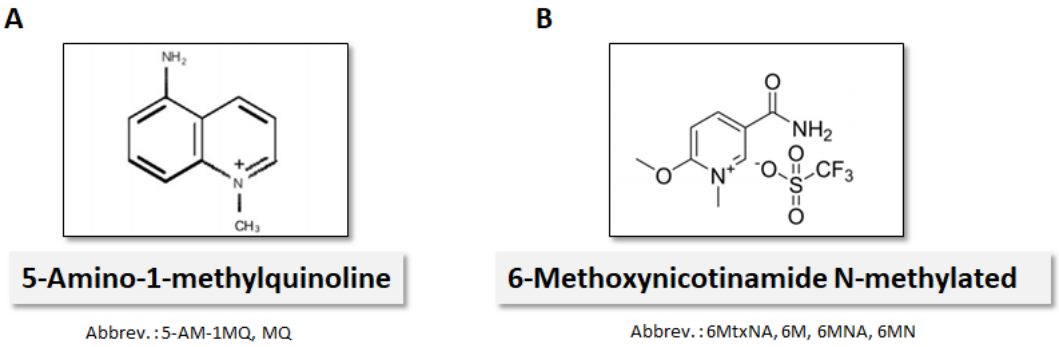


Figure 5. NNMT Inhibitors. Chemical structure of NNMT inhibitors 5-Amino-1-methylquinoline (A) and 6-Methoxynicotinamide N-methylated (B).

2.4 GLYCOLYTIC AND MITOCHONDRIAL METABOLISM

2.4.1 Mitochondrial function

A Seahorse Bioscience XFe96 Analyzer was used to measure mitochondrial function in adherent endothelial EAhy.926 and HAEC cells.

Cells were seeded into Seahorse XF96-well plates 24 h before the experiment, according to the Seahorse protocol. A confluence of 90% per well was selected as ideal. One day before the experiment, sensor cartridges were hydrated in XF calibrant and maintained at 37°C in air without CO_2. On the day of the experiment, cells were washed once and incubated with bicarbonate-free low-buffered assay medium (glucose 1 g/l, GlutaMAX 2 mM, sodium pyruvate 1 mM, pH 7.4) for one hour at 37°C in the absence of CO_2 prior to the beginning of the assay. Changes in cellular respiration were assessed over time in a mitochondrial functional assay where there was a sequential injection of reagents: oligomycin 1 µg/ml in port A followed by addition of 0.7 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) in port B and 1 µM rotenone/antimycin A in port C.

From the mitochondrial assay, the following parameters were determined: oxygen consumption rate (OCR), ATP-linked respiration, proton leak, maximal respiration, reserve capacity, non-mitochondrial respiration using the Seahorse-XF 96 (Seahorse Bioscience, North Billerica, MA, USA). Finally, data were analyzed using Seahorse Wave.

2.4.2 Glycolytic function in EAhy.926 NNMT silenced lines

Cells were seeded into Seahorse XF96-well plates 24 h before the experiment according to the Seahorse protocol. A confluence of 90% per well was selected as ideal. The day of experiment, cells were treated with drugs at various time points according to the Seahorse Glycolysis Stress Test protocol. Extracellular acidification rate (ECAR) in the glycolysis stress test was determined using the Seahorse-XF 96 (Seahorse Bioscience, North Billerica, MA, USA), and data were analyzed using Seahorse Wave.

2.5 CELL VIABILITY

2.5.1 Mitochondria quantification

Number of mitochondria was calculated using MitoSOX™ (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to manufacturer protocol. Briefly, cells were seeded into CQ1 96-well plates 24 h before the experiment. A confluence of 80% per well was selected as ideal for the experiment. Each well was stained with 50 µl of 5 µM MitoSOX™ reagent working solution for 10 minutes at 37˚C, protected from light. Wells were then washed gently twice by PBS 1X and left in 50 µl of PBS 1X for imaging at CQ1 confocal quantitative image cytometer (Yokogawa, Musashino, Tokyo, Japan), using CQ1 1.04 software. Images of MitoSOX- stained cells were then analyzed automatically by Columbus 2.4.2 software (Perkin Elmer, Waltham, MA, USA).

2.5.2 MTS assay

In order to assess cell viability and proliferation rate, MTS assay (Abcam, Cambridge, UK) was performed.

To assess the cell viability after stressor-induced injury, the day prior to the treatment cells were seeded (1 x 10^4) in a 96-well plate in a final volume of 100 µl/well of complete medium. The day after, cells were washed with PBS 1X, medium was replaced with fresh one, and cells were treated with increasing concentrations of H_2O_2 or $ONOO^-$, ranging between 10 and 1000 µM, and incubated for 24 h. According to manufacturer protocol, on the day of measurement 20 µl of MTS Reagent was added to each well and the plate was incubated for 1 hour at 37°C in standard culture conditions. Finally, it was measured the absorbance using a plate reader at OD=490 nm.

To determine the proliferation rate, cells were seeded (1 x 10^4) in a 96-well plate in a final volume of 100 µl/well of complete medium in the presence of 6-Methoxynicotinamide N-methylated 30 µM. At each timepoint (0, 24, 48, 72, 96 h), it was performed a MTS assay as previously described. Results were shown as ΔOD at 490 nm, obtained from the difference between the absorbance values at timepoints 24, 48, 72 and 96, and that at timepoint 0.

2.5.3 Cell counting

Cells were stained using YO-PRO™-1 Iodide (Thermo Fisher Scientific, USA). Cells were washed by PBS 1X, and incubated with a solution of 4% paraformaldehyde at room temperature. Subsequently, cells were washed by PBS 1X, and incubated with a solution of YO-PRO™-1 Iodide diluted 1:1000 in the dark, at 37°C. Finally, cells were washed twice by PBS 1X and left in PBS 1X for imaging at Confocal Quantitative

Image Cytometer (CQ1) (Yokogawa, Musashino, Tokyo, Japan). Images were analyzed through Columbus 2.4.2 software (Perkin Elmer, Waltham, MA, USA).

2.5.4 UV-C treatment

Cells were seeded in 6-well plates (1.5 x 10^5 cells/well) the day before the experiment. After 24 hours, medium was removed and cells were washed twice by PBS 1X. Cells were then incubated with 500 µl HBSS buffer and irradiated by UV-C light for 0, 3, 5, 7, 10, 15 min.

2.5.5 DHE fluorescence

For the measurement of superoxide generation, cells were incubated in HBSS buffer with DHE (dihydroethidinium) solution (1µM for 30 min at 37°C), according to producer's protocol (Thermo Fisher Scientific, USA), and then washed and fixed with 4% paraformaldehyde for 10 min. Cells were imaged using confocal quantitative image cytometer CQ1 (Yokogawa, Musashino, Tokyo, Japan) and images were analyzed automatically through Columbus 2.4.2 software (Perkin Elmer, Waltham, MA, USA).

2.5.6 FITC Annexin V test

FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, USA) was used to determine the percentage of apoptotic and necrotic cells, according to manifacturer's protocol. Cells were detached using Accutase® solution (Saint Louis, Missouri, USA) and centrifuged at 500 g for 5 min. Supernatant was discarded and pellets were washed twice by cold PBS and then resuspended in 1X Binding Buffer at a concentration of 1 x

10^6 cells/ml. 100 µl of the cellular suspension was transferred to a 5 ml culture tube adding 5 µl of FITC Annexin V and 5 µl propidium iodide (PI), and cells were incubated in the dark for 15 min at room temperature. After washing, Hoechst 33258 (Sigma Aldrich, Saint Louis, MO, USA) was used for nuclei staining (1µg/ml/10 min). Finally, 400 µl of 1X Binding Buffer was added to each tube and samples were analyzed by BD LSRII flow cytometer (BD Biosciences, San Jose, USA) within 1 h. For the detection of nuclei, apoptotic and necrotic cells, 405 nm violet laser, 488 nm blue laser and 561 nm yellow-green laser were used, respectively. Detected events were then analyzed by BD FACSDiva 6.1.3 software.

2.6 STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism software version 6.00 for Windows (GraphPad Prism Software, San Diego, CA, USA). Differences between groups were determined using the Mann-Whitney nonparametric test, or Kruskal-Wallis one-way ANOVA, followed by *post-hoc* Dunn test. A p-value < 0.05 was accepted as statistically significant.

3. RESULTS

3.1 NNMT SILENCING IN EAhy.926 CELLS

3.1.1 Efficiency of NNMT silencing

In order to modulate NNMT expression for functional assays, EAhy.926 line was stably transfected with empty pLKO.1 puro vector (pLKO.1 ∅), four shRNA plasmids targeting different regions of NNMT mRNA, or treated with transfection reagent only (mock), as described in the paragraph 2.1.2. The specific effects of shRNA treatment on NNMT expression were evaluated at both mRNA and protein levels by Real-Time PCR and Western blot.

Real-Time PCR showed a significant ($p<0.05$) downregulation of NNMT in cells transfected with pLKO.1-164 (2.98-fold reduction), pLKO.1-330 (3.04-fold reduction) pLKO.1-448 (3.71-fold reduction) and pLKO.1-711 (5.10-fold reduction) plasmids compared with control cells (Figure 6A). Empty pLKO.1 puro vector (pLKO.1 ∅) did not affect NNMT expression in a significant way (0.03-fold reduction). Therfore, further experiments were performed using either untreated EAhy.926 or empty pLKO.1 puro vector-transfected cells.

Results obtained by quantitative Real-Time PCR were confirmed at protein level by Western blot analysis. Lanes loaded with equal protein amounts displayed markedly decreased NNMT expression in cells transfected with shRNA-encoding vectors compared to controls (Figure 6B).

A LC/MS/MS-based assay was performed to measure MNA production. In keeping with the results of Real-Time PCR and Western blot, the levels of MNA were significantly lower in all NNMT knockdown cell lines compared to control (Figure 7).

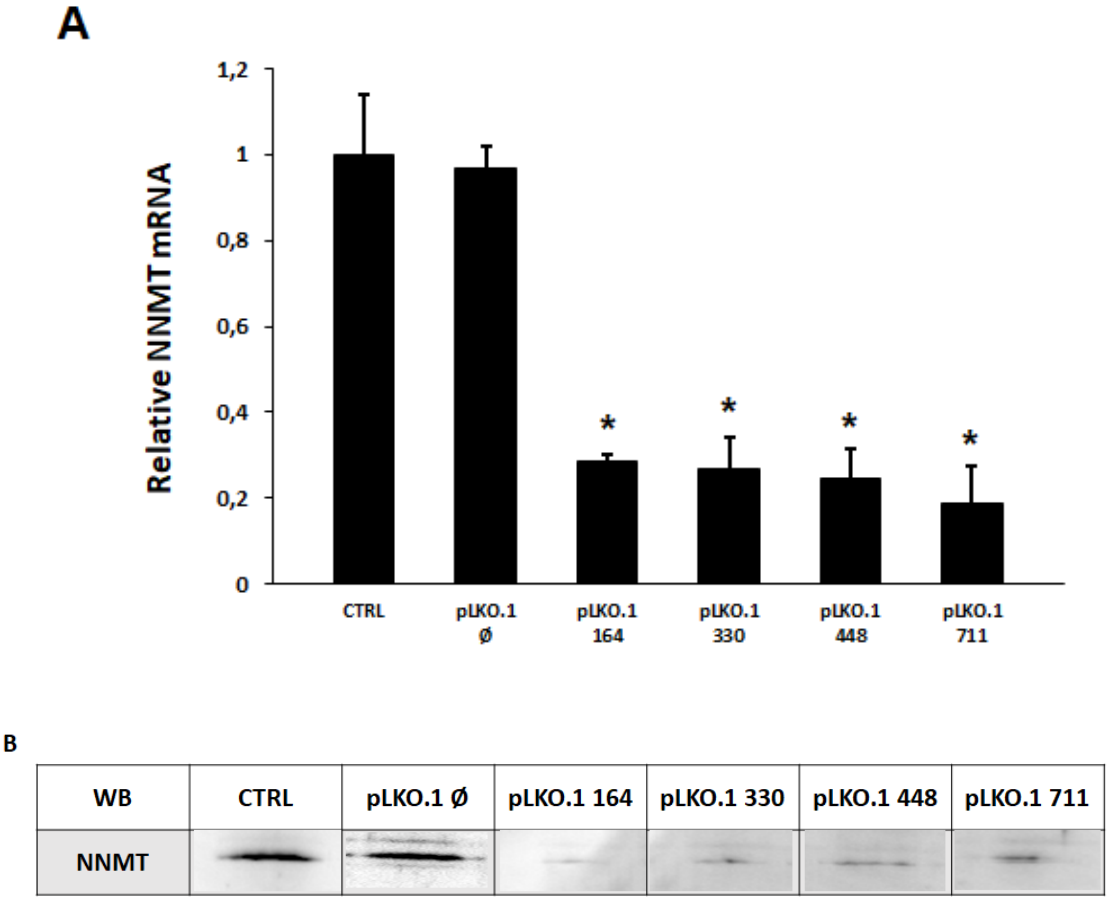


Figure 6. Evaluation of NNMT silencing. NNMT expression levels were analyzed in EAhy.926 cells transfected with empty pLKO.1 puro vector (pLKO.1 Ø), 4 shRNA plasmids (pLKO.1-164, 1-330, 1-448 and 1-711) or treated with transfection reagent only (mock), by Real-Time PCR (A) and Western Blot (B). Values are reported as mean ± standard deviation (* $p < 0.05$).

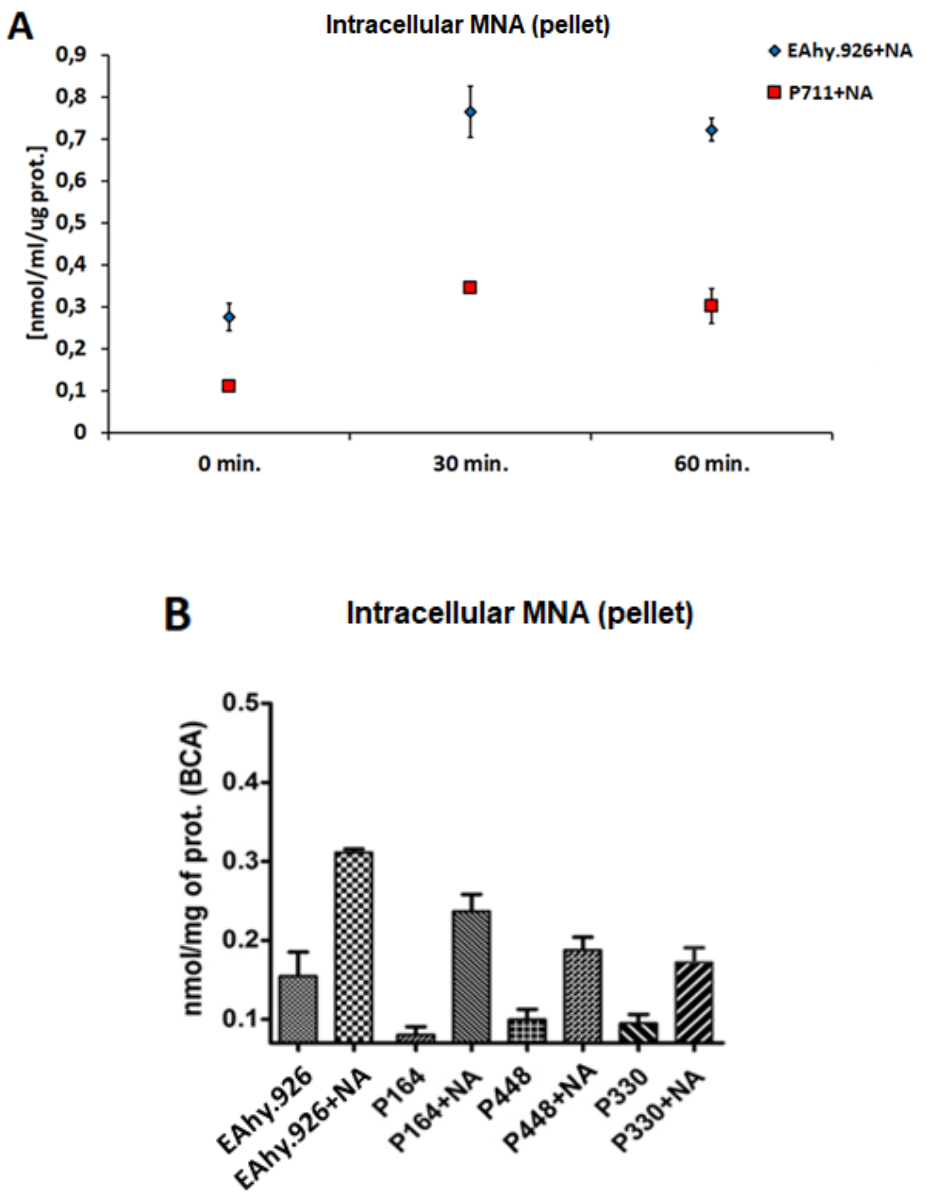
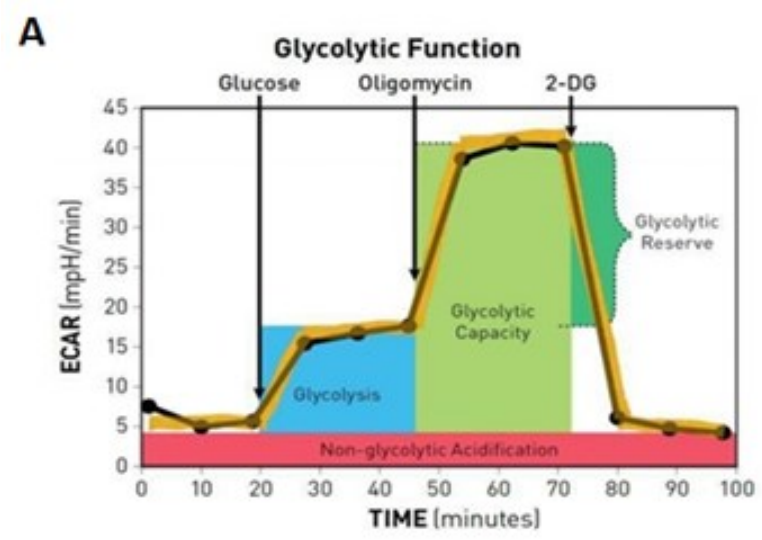


Figure 7. MNA production in NNMT knockdown cell lines. Intracellular MNA levels were analyzed in normal EAhy.926 cells and EAhy.926 cells transfected with 4 shRNA plasmids: pLKO.1- 1-711 (A) and pLKO.1-164, 1-330, 1-448 (B). In both cases cells were incubated with nicotinamide 100 µM for the entire duration of the experiment. Intracellular MNA was detected by LC/MS/MS at timepoints 0, 30, 60 min for P711 line, and at timepoint 60 min for all other lines. Values are reported as mean ± standard deviation (* $p < 0.05$).

3.2 GLYCOLYTIC AND MITOCHONDRIAL METABOLISM IN CELL LINES AFTER NNMT KNOCKDOWN

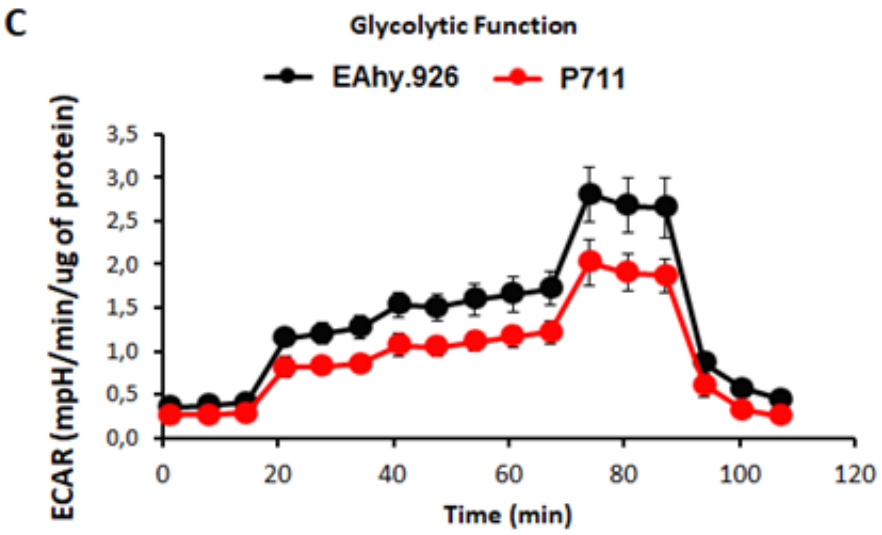
3.2.1 Glycolytic function in EAhy.926 and NNMT silenced lines

The effect of NNMT silencing on glycolysis *in vitro* was examined by monitoring the extracellular acidification rate (ECAR) in the glycolysis stress test by Seahorse Technology (Figure 8). In the silenced lines transfected with 4 shRNA plasmids (pLKO.1-164, 1-330, 1-448 and 1-711) the glycolytic function seems to be marginally downregulated, although not in a significant way.



B

Parameter	Rate Measurement Equation Used by Report Generator
Glycolysis	(Maximum rate measurement before Oligomycin injection) – (Last rate measurement before Glucose injection)
Glycolytic Capacity	(Maximum rate measurement after Oligomycin injection) – (Last rate measurement before Glucose injection)
Glycolytic Reserve	(Glycolytic Capacity) – (Glycolysis)
Glycolytic Reserve as a %	(Glycolytic Capacity Rate) /(Glycolysis) × 100
Non-Glycolytic Acidification	Last rate measurement prior to glucose injection
Acute Response	(Last measurement rate before glucose injection – Last rate measurement before acute injection)



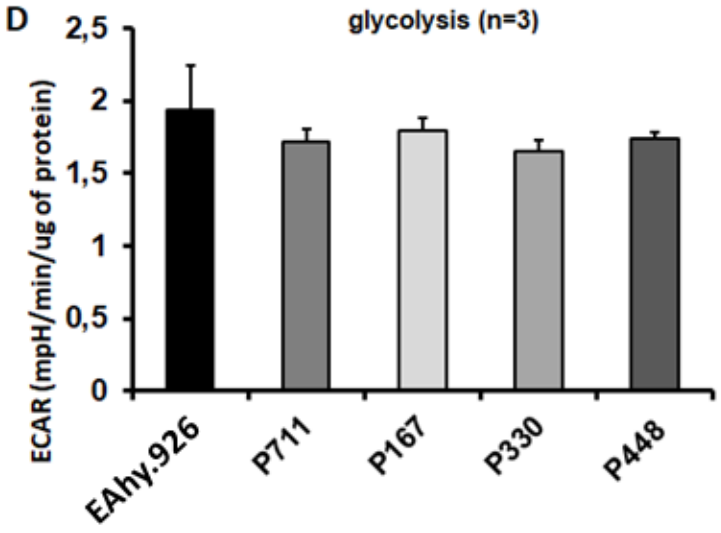


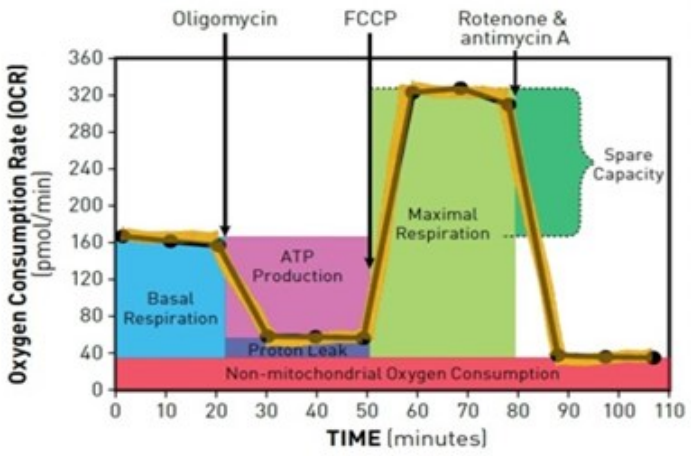
Figure 8. Assessment of NNMT silencing on glycolysis. Agilent Seahorse XF Glycolysis Stress Test profile of the key parameters of glycolytic function (A) and (B).

Glycolytic function profile in P711 line (C) and glycolysis levels in all NNMT silenced lines P711, P164, P330, P448 (D). Values are reported as mean ± standard deviation.

3.2.2 Mitochondrial respiration in EAhy.926 and NNMT silenced lines

The effect of NNMT silencing on mitochondrial respiration *in vitro* was examined by monitoring the oxygen consumption rate (OCR) through the Cell Mito Stress Test, by using Seahorse Technology. In the NNMT silenced lines transfected with 4 shRNA plasmids (pLKO.1-164, 1-330, 1-448 and 1-711) the mitochondrial respiration seems slightly downregulated (Figure 9). Basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity levels result decreased compared to control line EAhy.926 (Figure 10).

A



Parameter Value	Equation
Non-mitochondrial Oxygen Consumption	Minimum rate measurement after Rotenone/antimycin A injection
Basal Respiration	(Last rate measurement before first injection) – (Non-Mitochondrial Respiration Rate)
Maximal Respiration	(Maximum rate measurement after FCCP injection) – (Non-Mitochondrial Respiration)
H+ (Proton) Leak	(Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial Respiration
ATP Production	(Last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection)
Spare Respiratory Capacity	(Maximal Respiration) – (Basal Respiration)
Spare Respiratory Capacity as a %	(Maximal Respiration) / (Basal Respiration) × 100
Acute Response	(Last rate measurement before oligomycin Injection) – (Last rate measurement before acute injection)
Coupling Efficiency	ATP Production Rate) / (Basal Respiration Rate) × 100

B

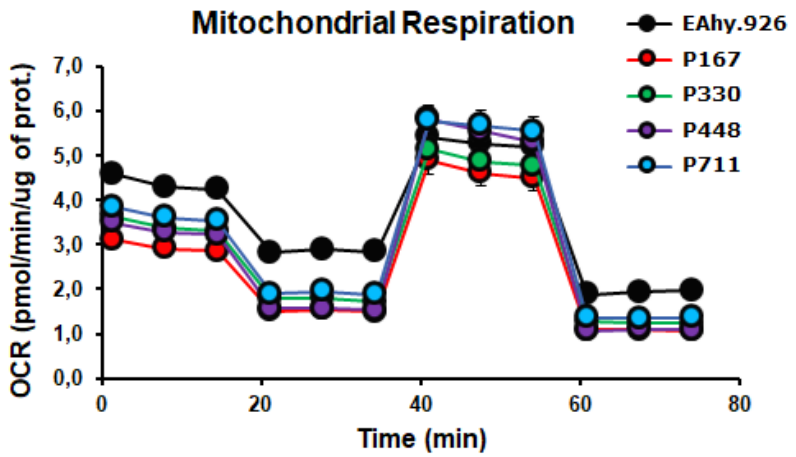
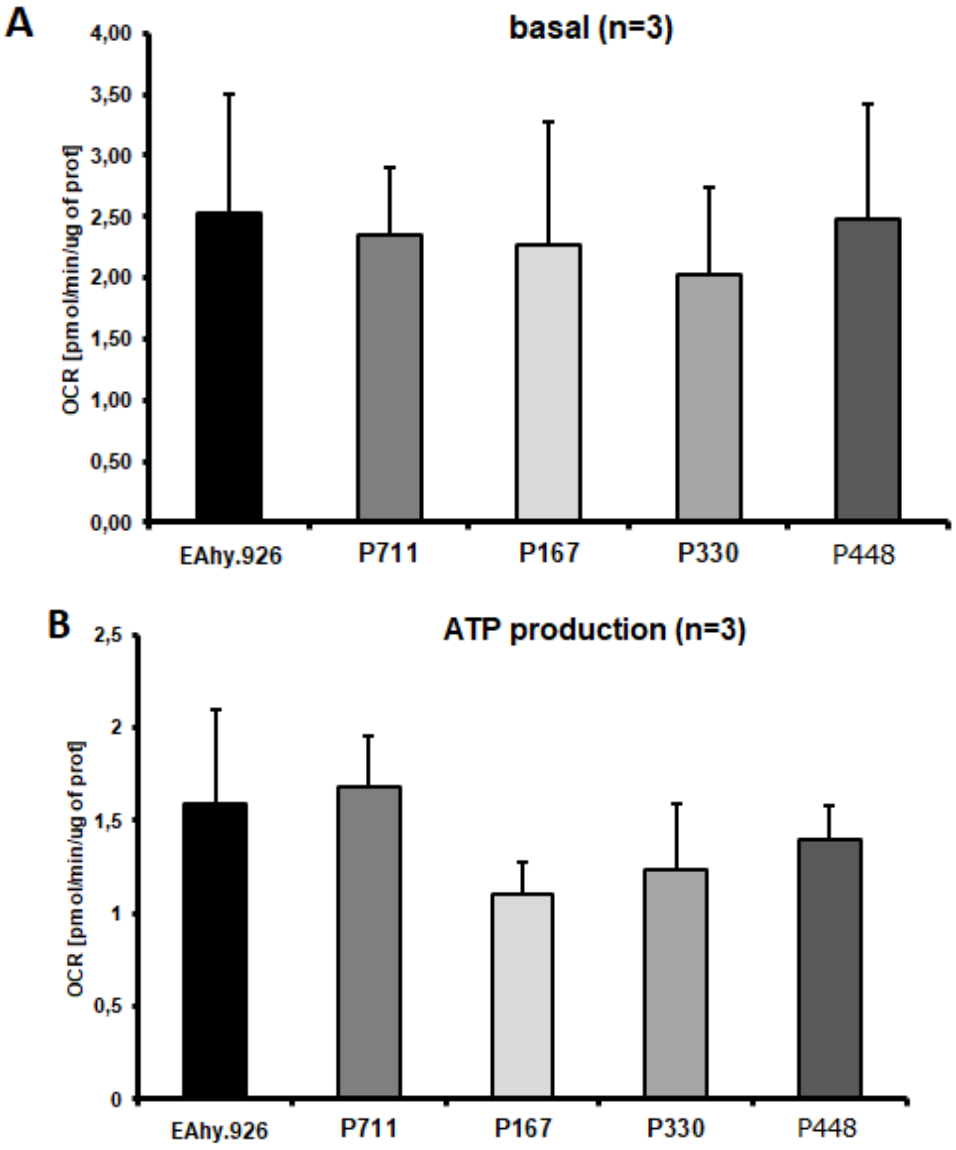


Figure 9. Seahorse XF Cell Mito Stress Test profile in EAhy.926 and NNMT silenced lines. Agilent Seahorse XF Cell Mito Stress Test profile of the key parameters

of mitochondrial respiration (A). OCR profile in NNMT silenced lines P167, P330, P448, P711 (B).



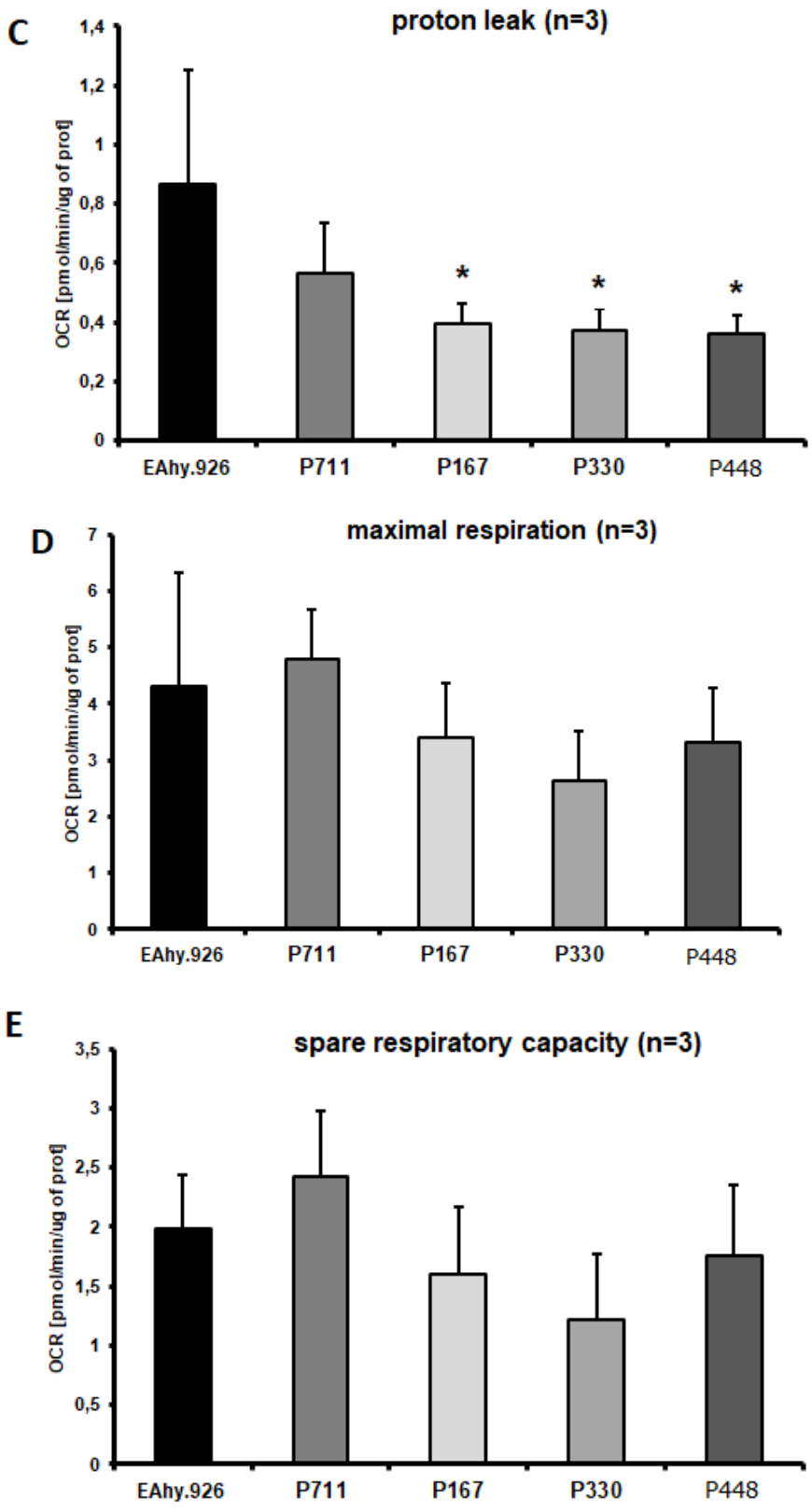
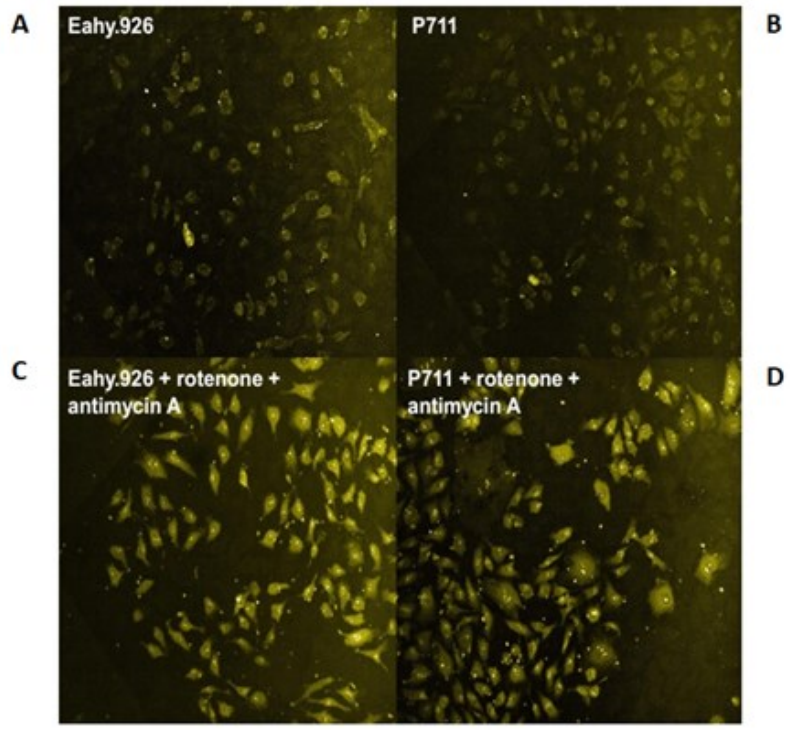


Figure 10. Seahorse XF Cell Mito Stress Test parameters in EAhy.926 and NNMT silenced lines. Agilent Seahorse XF Cell Mito Stress Test parameters in NNMT

silenced lines P167, P330, P448, P711. Basal respiration (A), ATP production (B), proton leak (C), maximal respiration (D), spare respiratory capacity (E). Values are reported as mean ± standard deviation (* $p < 0.05$).

3.2.3 Confocal imaging of MitoSOX-stained mitochondria in EAhy.926 and P711 NNMT silenced clone

To assess whether variations in the mitochondrial respiration observed in NNMT silenced cells could be a consequence of a different amount of mitochondria, it was calculated the number of mitochondria in EAhy.926 and P711 line using MitoSOX™. MitoSOX™ Red reagent permeates live cells and selectively targets mitochondria. Once oxidized by superoxide, but not by other reactive oxygen species (ROS) and reactive nitrogen species (RNS), it results highly fluorescent upon binding to nucleic acid. The number of mitochondria observed in EAhy.926 compared to P711 NNMT silenced line was not significantly different (Figure 11).



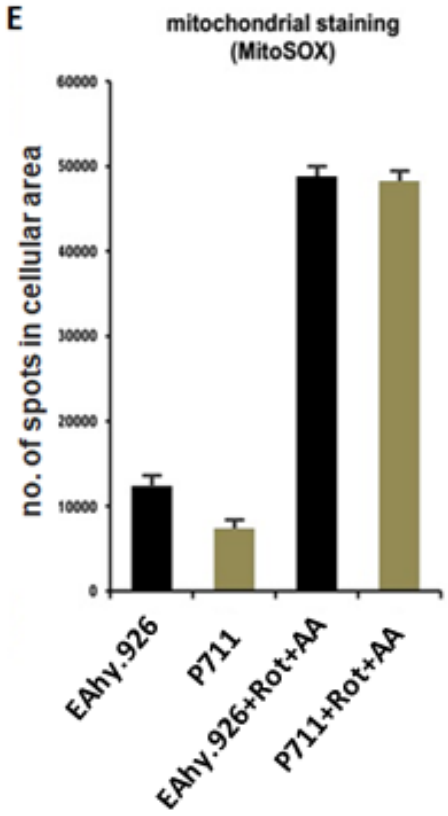


Figure 11. Confocal imaging of MitoSOX-stained mitochondria in EAhy.926 and P711 NNMT silenced line. Mitochondria number was analyzed by MitoSOX™ Red reagent in EAhy.926 (A and C) and P711 cells (B and D), treated (C and D) or not (A and B) with rotenone and antimicin A. Number of spots detected in cellular area (E).

3.3 EFFECT OF OXIDATIVE AND GENOTOXIC STRESS ON CELLULAR VIABILITY AND APOPTOTIC PATHWAY IN NNMT SILENCED CELL LINES

3.3.1 Effect of NNMT knockdown cell viability of P711 clone on after menadione treatment

In order to test the effect of NNMT knockdown on oxidative stress response, normal EAhy.926 cells and P711 line were incubated with increasing concentrations of menadione for 24 h and then the cell viability was evaluated using YO-PRO™-1 Iodide stain. Menadione 50 μM induced a significant drop in cell viability of P711 line compared to control (Figure 12), suggesting a protective role for the enzyme in the endothelium. Subsequently, experiments were repeated with all NNMT silenced cell lines using as control the EAhy.926 line transfected with empty vector (pLKO.1 puro) (Figure 13). Data obtained confirmed the protective role of the enzyme in menadione-induced injury.

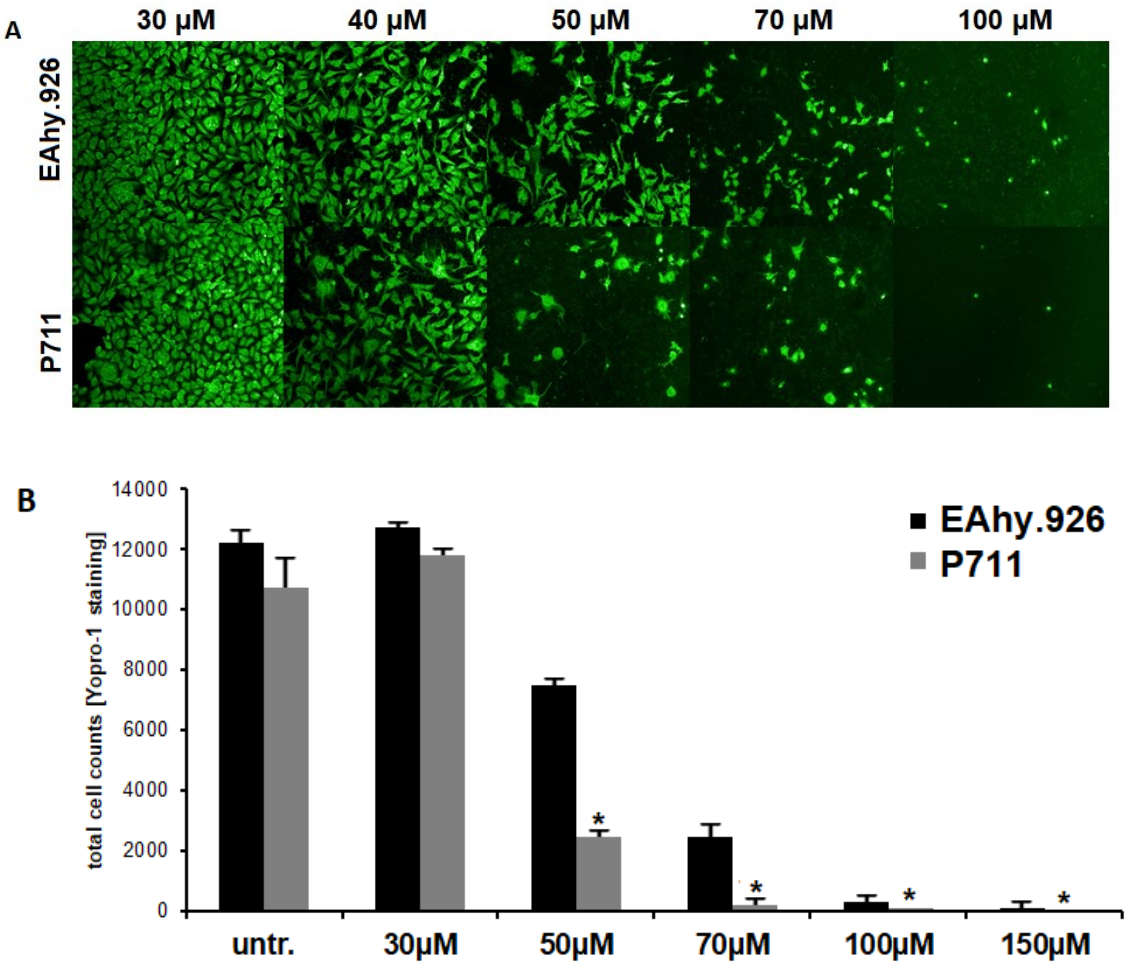


Figure 12. Effect of menadione treatment on cell viability in NNMT silenced cell line P711. EAhy.926 and P711 cell lines were incubated with increasing concentrations of menadione for 24 h and then the cell viability was evaluated by using YO-PRO™-1 Iodide stain. 20x magnification pictures (A) and total cell counts (B). Values are reported as mean ± standard deviation (* $p < 0.05$).

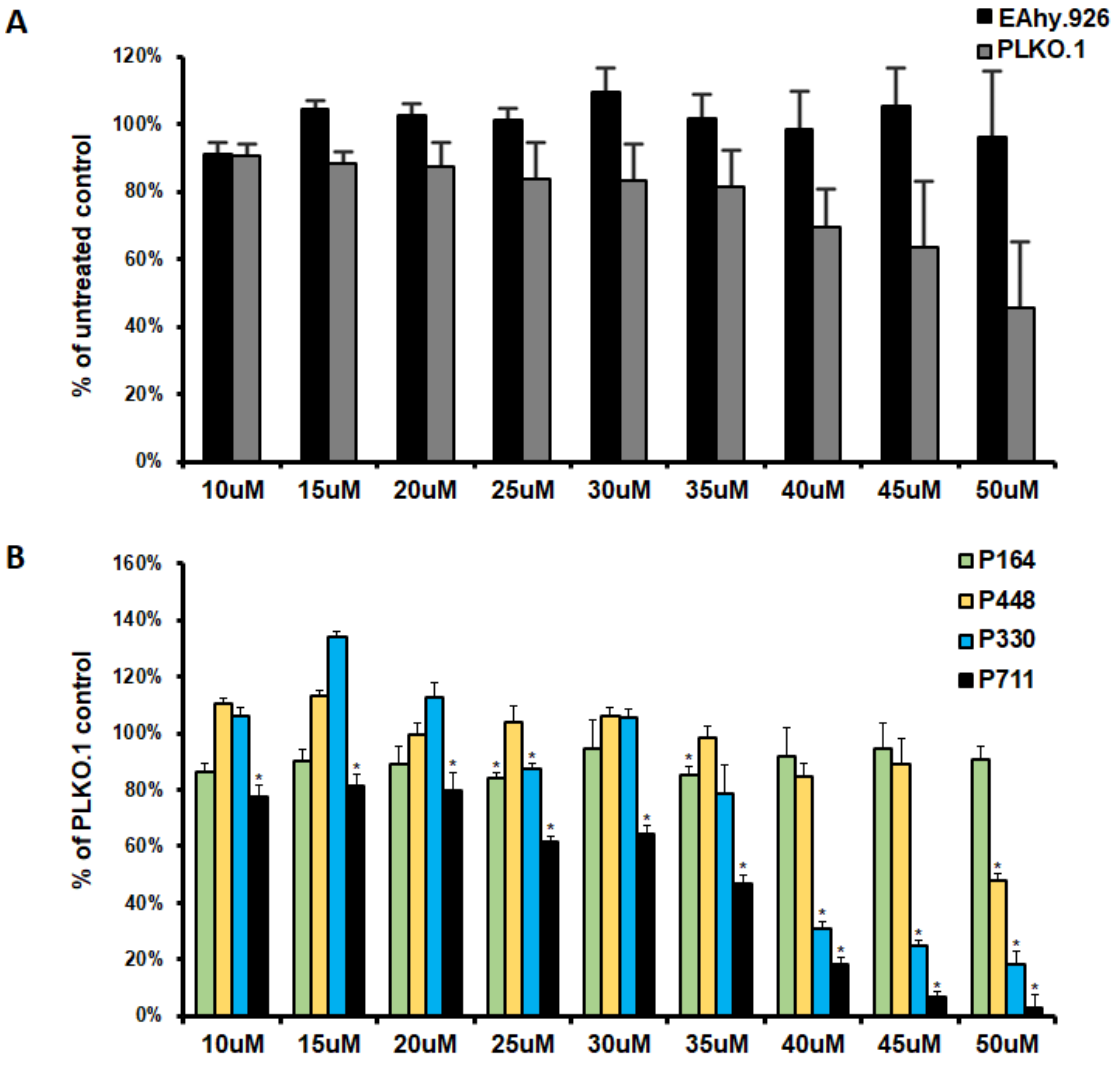
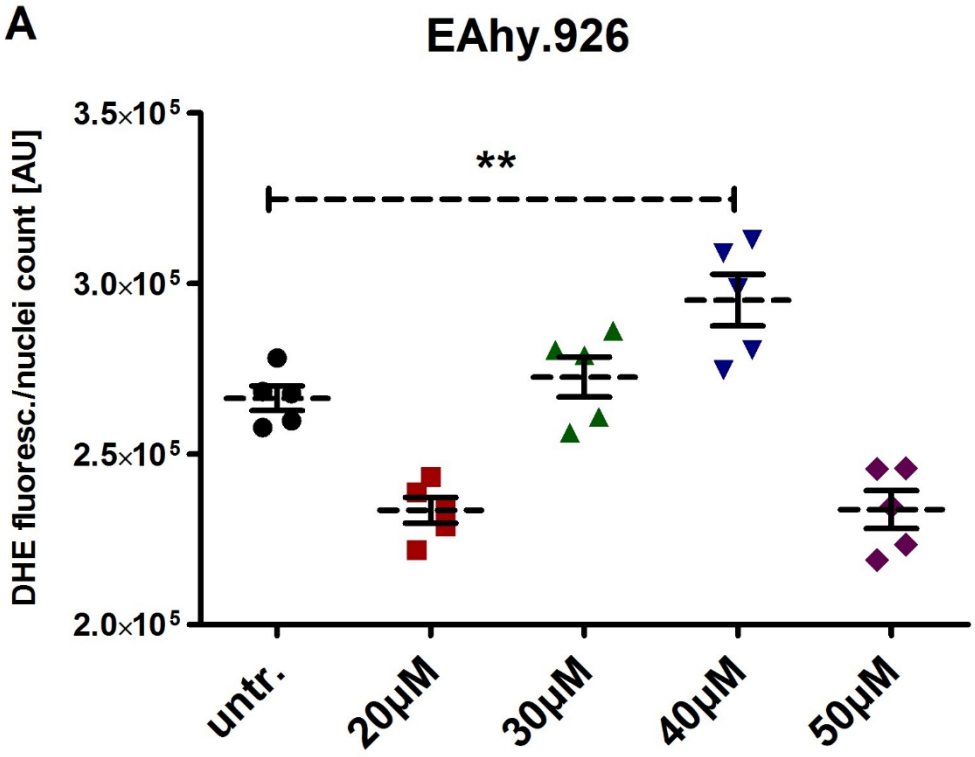


Figure 13. Effect of menadione treatment on cell viability in all NNMT silenced cell lines and pLKO.1 puro-treated line. EAhy.926 cells transfected with empty vector pLKO.1 puro (A) and all NNMT silenced cell lines (B) were incubated with increasing concentrations of menadione for 24 h and then the cell viability was evaluated using with YO-PRO™-1 Iodide stain. Values are reported as mean ± standard deviation (* $p < 0.05$).

3.3.2 ROS generation in NNMT knockdown cell line P711 after menadione treatment

In order to explore whether increased sensitivity to menadione injury in P711 line could be linked only to higher ROS production, it was analyzed the superoxide generation by the DHE fluorescence. Cells were incubated in HBSS buffer with DHE solution (1µM for 30 min at 37°C), then washed and fixed with 4% paraformaldehyde for 10 min. Cells were then imaged using confocal quantitative image cytometer CQ1 (Figure 14). Despite a moderate increase in ROS production in P711 line compared to control, the higher ROS levels in NNMT silenced lines cannot fully explain the increased menadione-induced sensitivity.



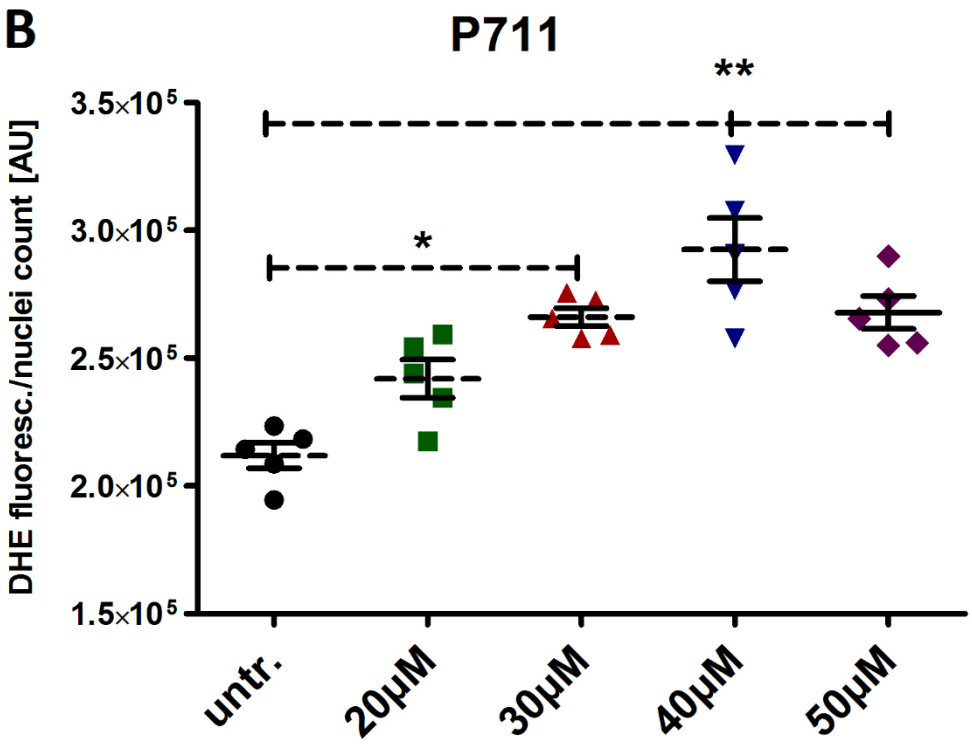


Figure 14. DHE fluorescence in EAhy.926 cells and P711 line. Menadione-induced ROS production was assessed in EAhy.926 cells (A) and P711 line (B), by measuring DHE fluorescence. (* $p < 0.05$, ** $p < 0.01$).

3.3.3 Effect of NNMT knockdown on cell viability after H_2O_2 treatment

To confirm the effect of NNMT knockdown on oxidative stress response, untreated EAhy.926 cells and NNMT silenced P711 line were incubated with increasing concentrations of H_2O_2 for 24 h and then the cell viability was evaluated by MTS assay. Data obtained revealed that P711 line was more sensitive to injury induced by H_2O_2 than control (Figure 15), especially for very high concentrations of the compound.

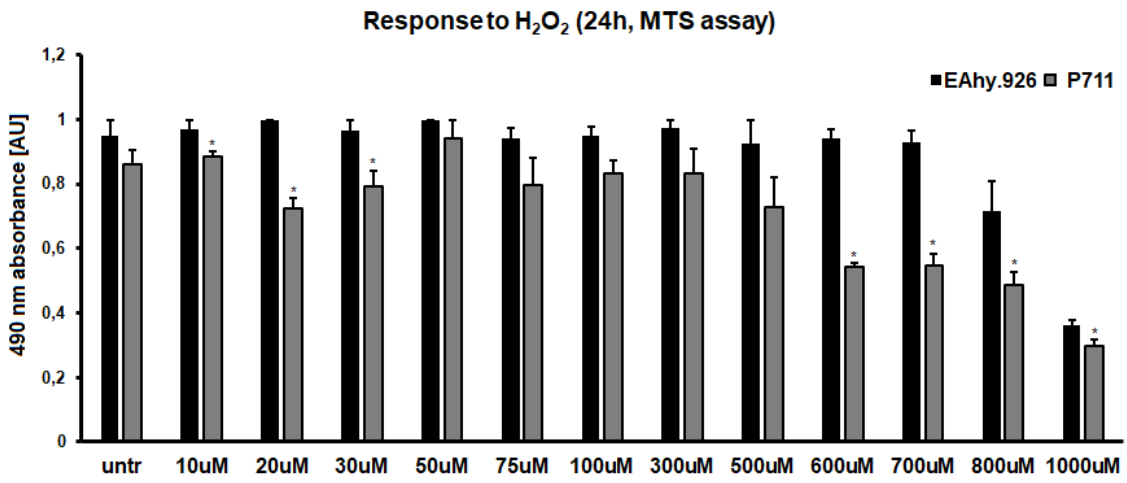


Figure 15. Effect of H_2O_2 treatment on cell viability in P711 NNMT silenced cell line. Cells were incubated with increasing concentrations of H_2O_2 for 24 h and then the cell viability was evaluated by MTS assay. Values are reported as mean ± standard deviation (* $p < 0.05$).

3.3.4 Effect of NNMT knockdown on cell viability after $ONOO^-$ treatment

Control EAhy.926 cells and NNMT silenced P711 line were incubated with increasing concentrations of $ONOO^-$ for 24 h and then the cell viability was evaluated by using YO-PRO™-1 Iodide stain (Figure 16). In line with data obtained with hydrogen peroxide, P711 line was shown to be more sensitive to injury induced by $ONOO^-$ than control, also for low concentrations of the stressor.

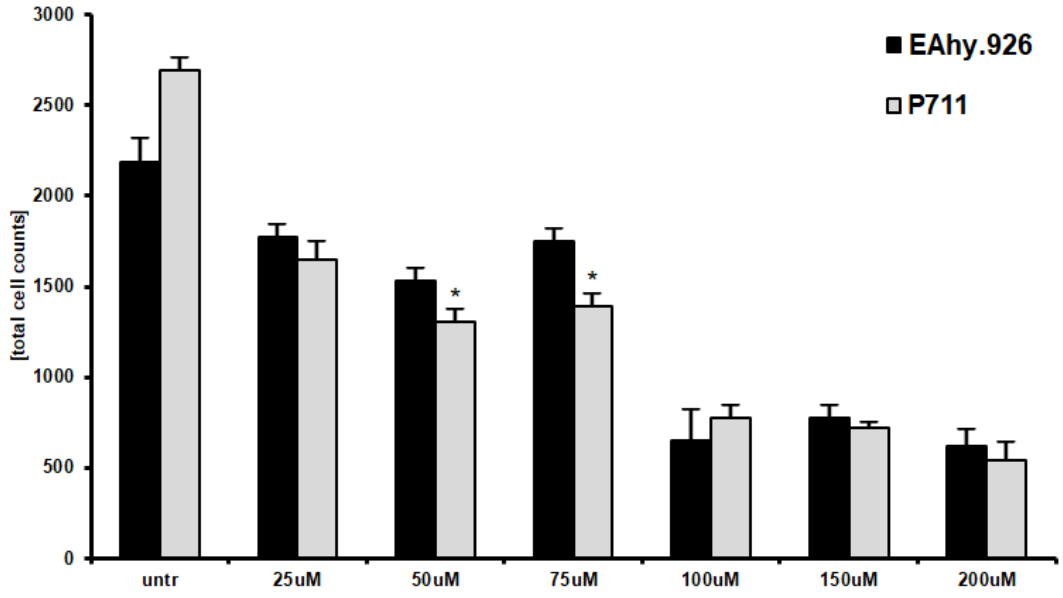


Figure 16. Effect of $ONOO^-$ treatment on cell viability in P711 NNMT silenced cell line. Total cell counts of EAhy.926 and NNMT silenced P711 cell lines after 24 h $ONOO^-$ treatment. Values are reported as mean ± standard deviation (* $p < 0.05$).

3.3.5 Effect of NNMT knockdown cell line P711 on cell viability after UV-C treatment

In order to assess whether NNMT silencing could have an effect on genotoxic injury, EAhy.926 cells and P711 line were irradiated by UV-C light for 3, 5, 7, 10 and 15 min and then stained by YO-PRO™-1 Iodide. P711 line showed an increased sensitivity to UV-C radiation compared to the control (Figure 17), suggesting that the enzyme could be involved, at least in an indirect way, in the DNA damage response.

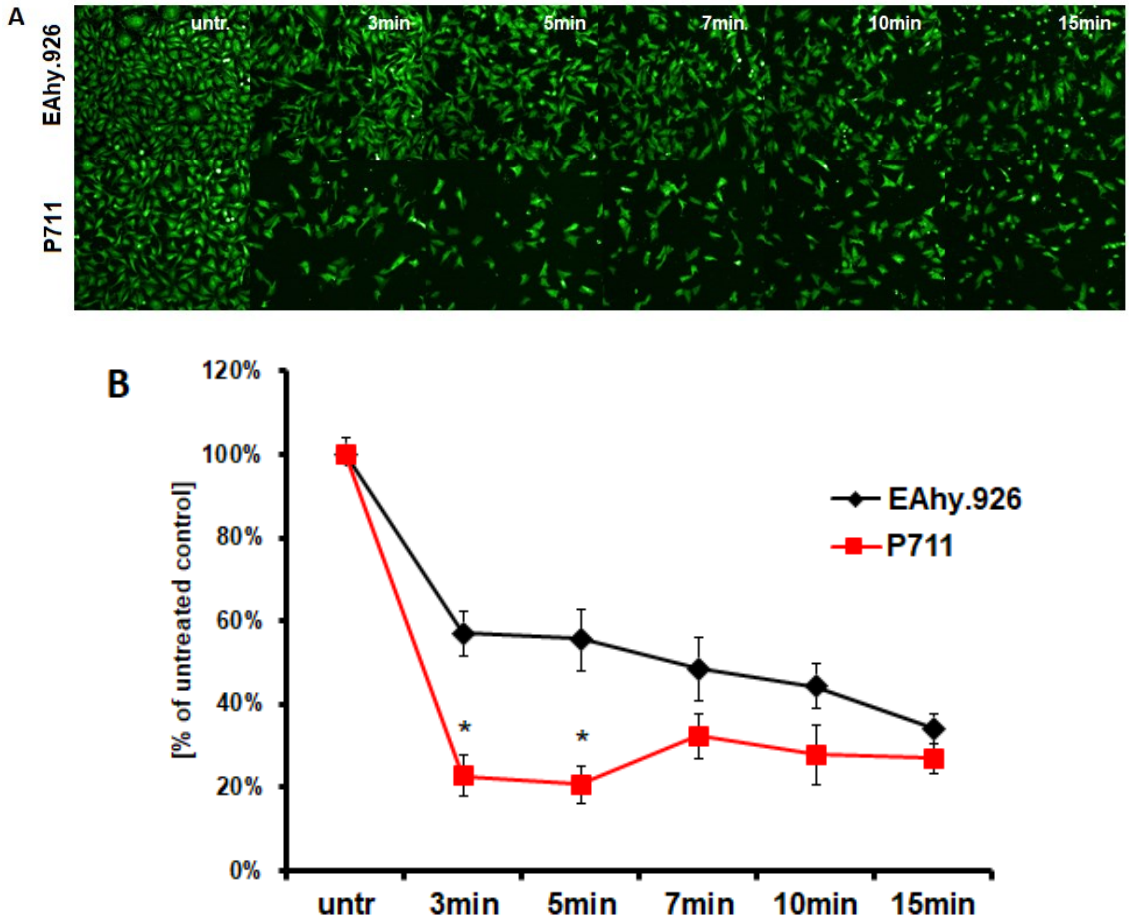


Figure 17. Effect of UV-C radiation on cell viability in P711 NNMT silenced cell line. EAhy.926 cells and P711 line were irradiated by UV-C light for 3, 5, 7, 10 and 15 min and then stained by YO-PRO™-1 Iodide. 20x magnification pictures (A) and percentage of living cells compared to control after the radiation (B). Values are reported as mean ± standard deviation (* $p < 0.05$).

3.3.6 Effect of NNMT silenced cell line P711 on apoptotic/necrotic rate after $ONOO^-$ treatment

In order to explore whether the increased $ONOO^-$-injury observed in the P711 line could be attributable to changes in the rate of apoptosis or necrosis, a cytometric Annexin

V/PI assay was run, after treating cells for 24 h with 30 and 50 µM peroxynitrite. Data obtained showed that peroxynitrite 30 µM induced the apoptosis in the 9.2% of P711 population compared to the 0.8% of the control, and the necrosis in 80.5% of P711 population compared to the 48.5% of the control (Figure 18). These results suggested that the reduced cell viability observed in NNMT silenced cell lines subjected to different stressors could be linked to an increase in apoptosis, necrosis, or both.

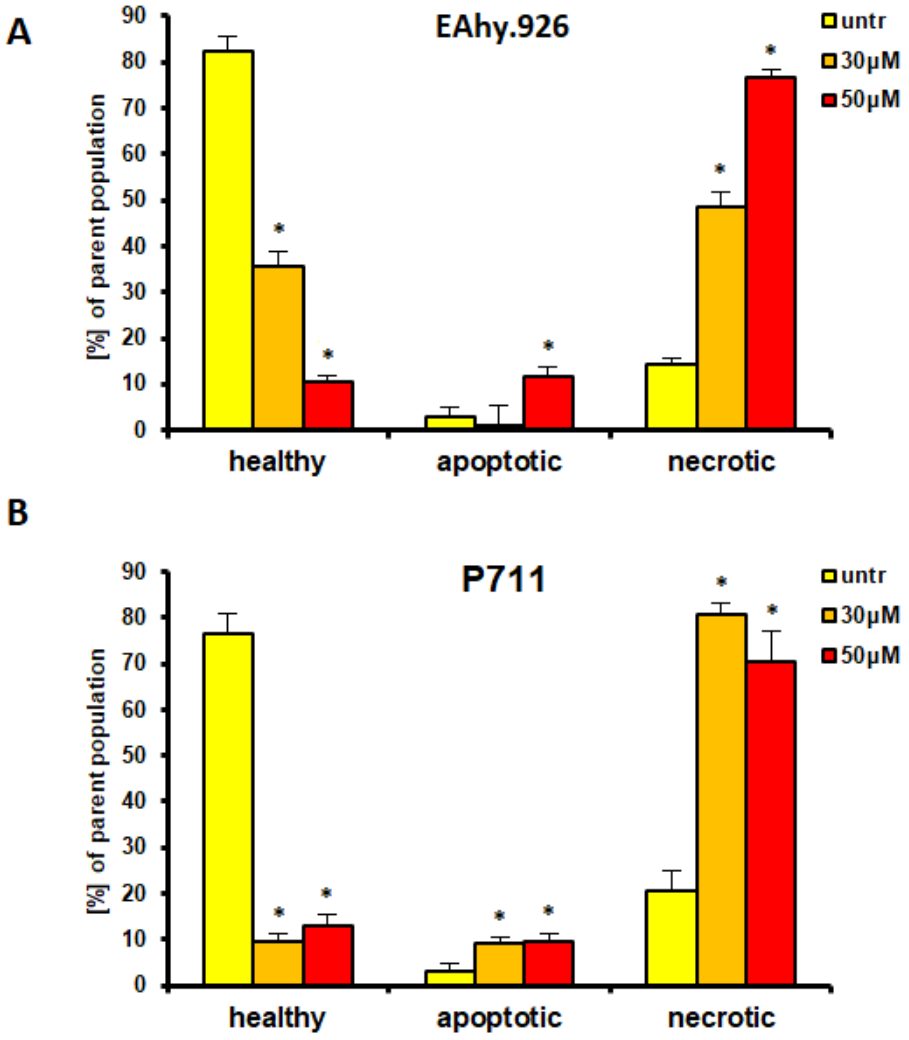


Figure 18. Effect of NNMT knockdown in cell line P711 on apoptotic/necrotic rate after $ONOO^-$ treatment. EAhy.926 and P711 cells were incubated for 24 h with

peroxynitrite (30 and 50 μM), and apoptosis/necrosis were estimated by cytometric Annexin V/PI assay. Values are reported as mean ± standard deviation (* $p < 0.05$).

3.4 NNMT INHIBITION IN HAEC CELL LINES

3.4.1 Effect of NNMT inhibition by novel inhibitors in mice liver

To test the efficiency of NNMT inhibitors, it was performed an *ex vivo* assay using mice liver samples. Aliquots of mice liver (20 mg) were incubated in HBSS buffer with nicotinamide 100 μM. At 0, 0.5, 1, 2, 3 h timepoints, an aliquot of extracellular fluid was collected and MNA concentration was analyzed by LC/MS/MS (Figure 19). 6-Methoxynicotinamide N-methylated seemed to be more potent than 5-Amino-1-methylquinoline. 6-Methoxynicotinamide N-methylated >10μM and 5-Amino-1-methylquinoline >30-50μM induced NNMT inhibition >90%.

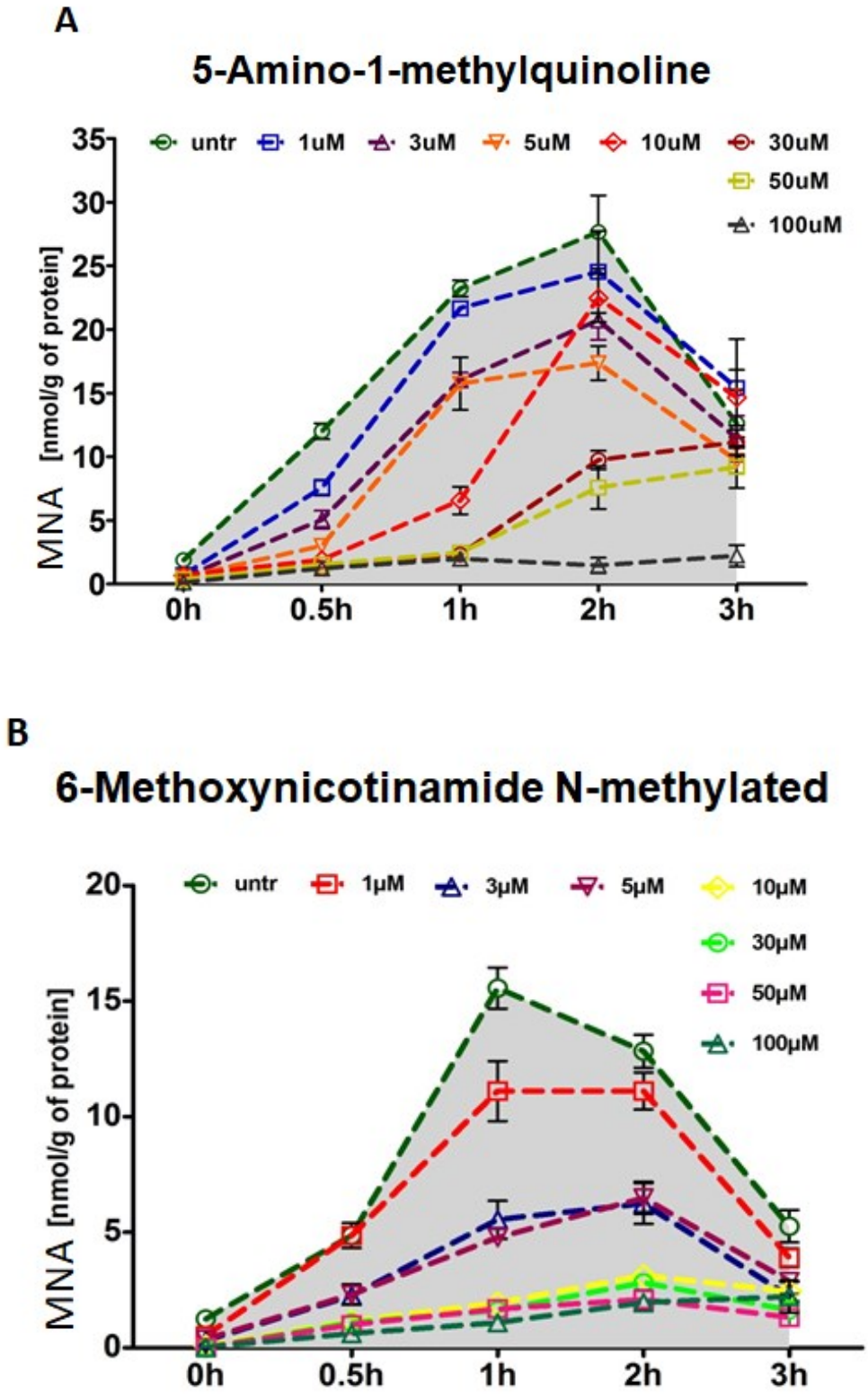
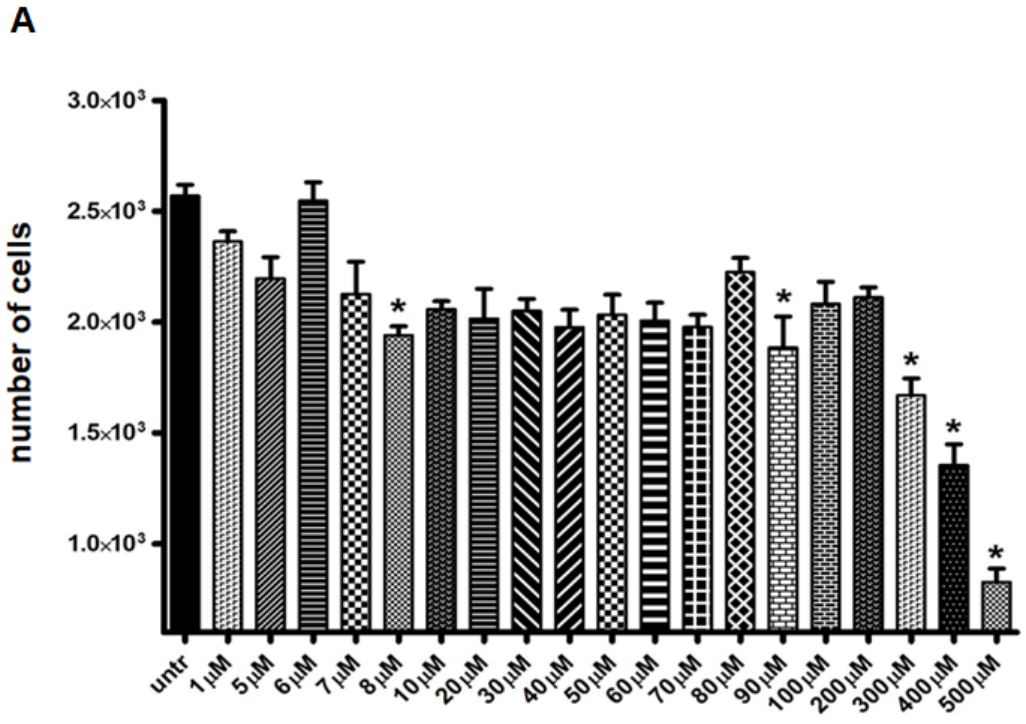


Figure 19. Efficiency of NNMT inhibitors on mice liver. Aliquots of mice liver (20 mg) were incubated in HBSS buffer with nicotinamide 100 μM and 5-Amino-1-

methylquinoline (A) or 6-Methoxynicotinamide N-methylated (B). Values are reported as mean ± standard deviation (* $p < 0.05$).

3.4.2 Assessment of NNMT inhibitors toxicity in HAECs

In order to evaluate the toxicity of NNMT inhibitors on endothelial cells, HAECs were incubated for 24 h with increasing concentrations of inhibitors, and then the number of living cells was calculated after staining with YO-PRO™-1 Iodide. Both 5-Amino-1-methylquinoline and 6-Methoxynicotinamide N-methylated demonstrated to be safe up to very high concentrations (300 µM for 5-Amino-1-methylquinoline and 500 µM for 6-Methoxynicotinamide N-methylated), although the concentrations useful to inhibit the enzyme are much lower (Figure 20).



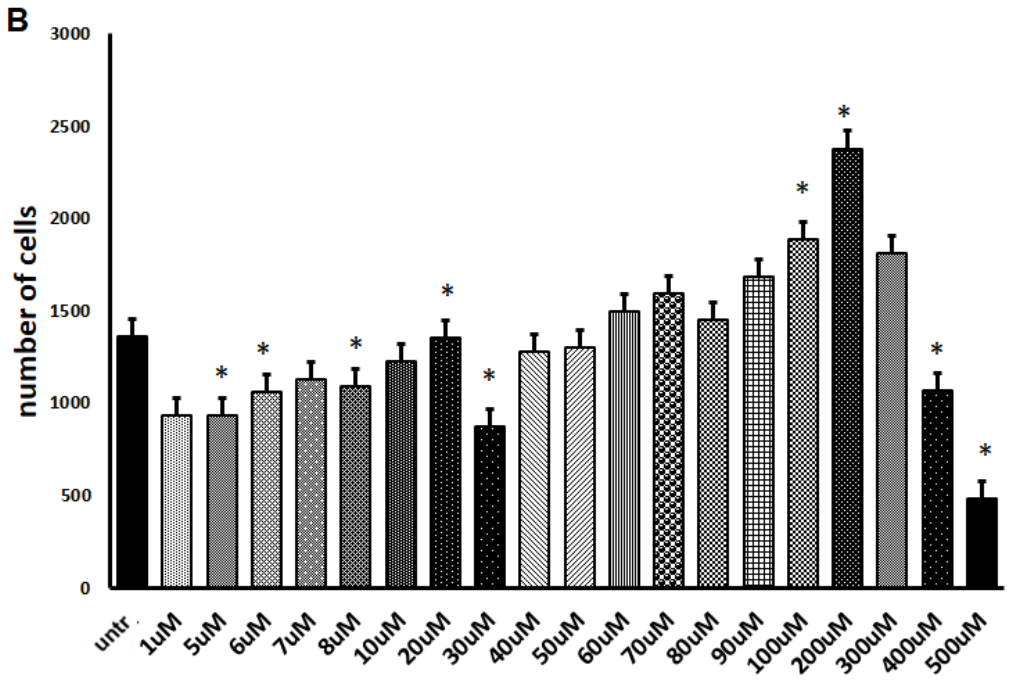


Figure 20. Effect of NNMT inhibitors on cell viability. Cell viability test on HAECs incubated for 24 h with 5-Amino-1-methylquinoline (A) and 6-Methoxynicotinamide N-methylated (B). Values are reported as mean ± standard deviation (* $p < 0.05$).

3.5 EFFECT OF OXIDATIVE STRESS ON CELLULAR VIABILITY IN NNMT INHIBITED HAECs

3.5.1 Effect of NNMT inhibition on HAECs viability

To confirm the data obtained on NNMT silenced cell lines, HAECs were incubated 24 h with different concentrations of menadione and 50 µM of 5-Amino-1-methylquinoline or 30 µM 6-Methoxynicotinamide N-methylated, alternatively. Subsequently, it was assessed the cell viability staining cells with YO-PRO™-1 Iodide. Similarly to what

happened for NNMT silenced cell lines, both inhibitors affected the cell viability, sensitizing the cells to the damage induced by menadione (Figure 21). The effect was more evident for 6-Methoxynicotinamide N-methylated, which demonstrated to have higher efficiency in inhibiting NNMT than 5-Amino-1-methylquinoline.

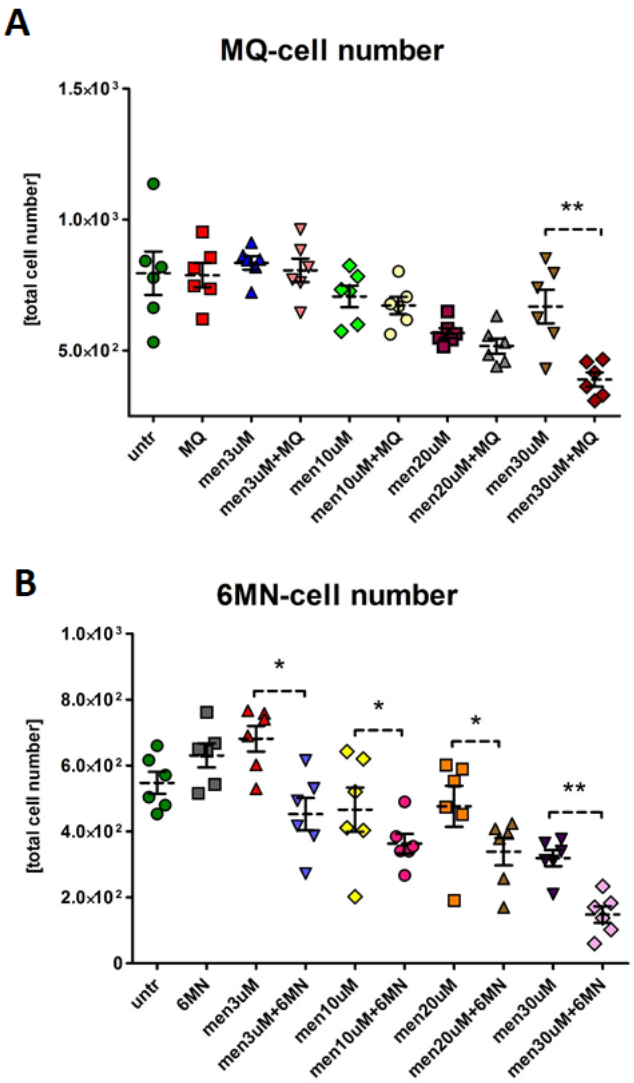


Figure 21. Effect of NNMT inhibition on cell viability after menadione-induced injury. HAECs were incubated 24 h with different concentrations of menadione together with 50 µM of 5-Amino-1-methylquinoline (MQ) (A) or 30 µM 6-Methoxynicotinamide N-methylated (6MN) (B) (* $p < 0.05$, ** $p < 0.01$).

3.6 MITOCHONDRIAL METABOLISM IN NNMT INHIBITED HAECs

3.6.1 Effect of NNMT inhibition induced by 5-Amino-1-methylquinoline on HAECs mitochondrial respiration

In order to verify data obtained in NNMT silenced cell lines, it was analyzed the mitochondrial respiration on primary HAECs in the presence of inhibitors (Figure 22). When incubated for 1 h with increasing concentrations of 5-Amino-1-methylquinoline, HAECs showed decreased basal respiration, ATP production, maximal respiration and spare respiratory capacity levels (Figure 23). These data are consistent with those obtained in NNMT silenced EAhy.926 cells, strengthening the hypothesis that NNMT can affect mitochondrial respiration in endothelial cells.

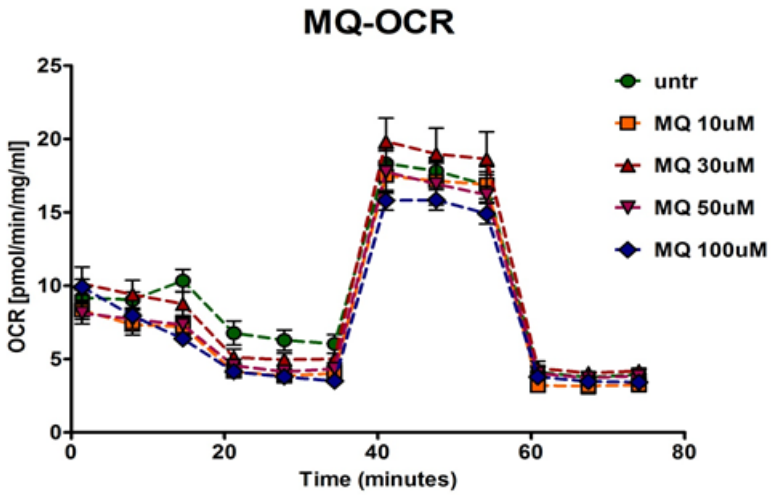
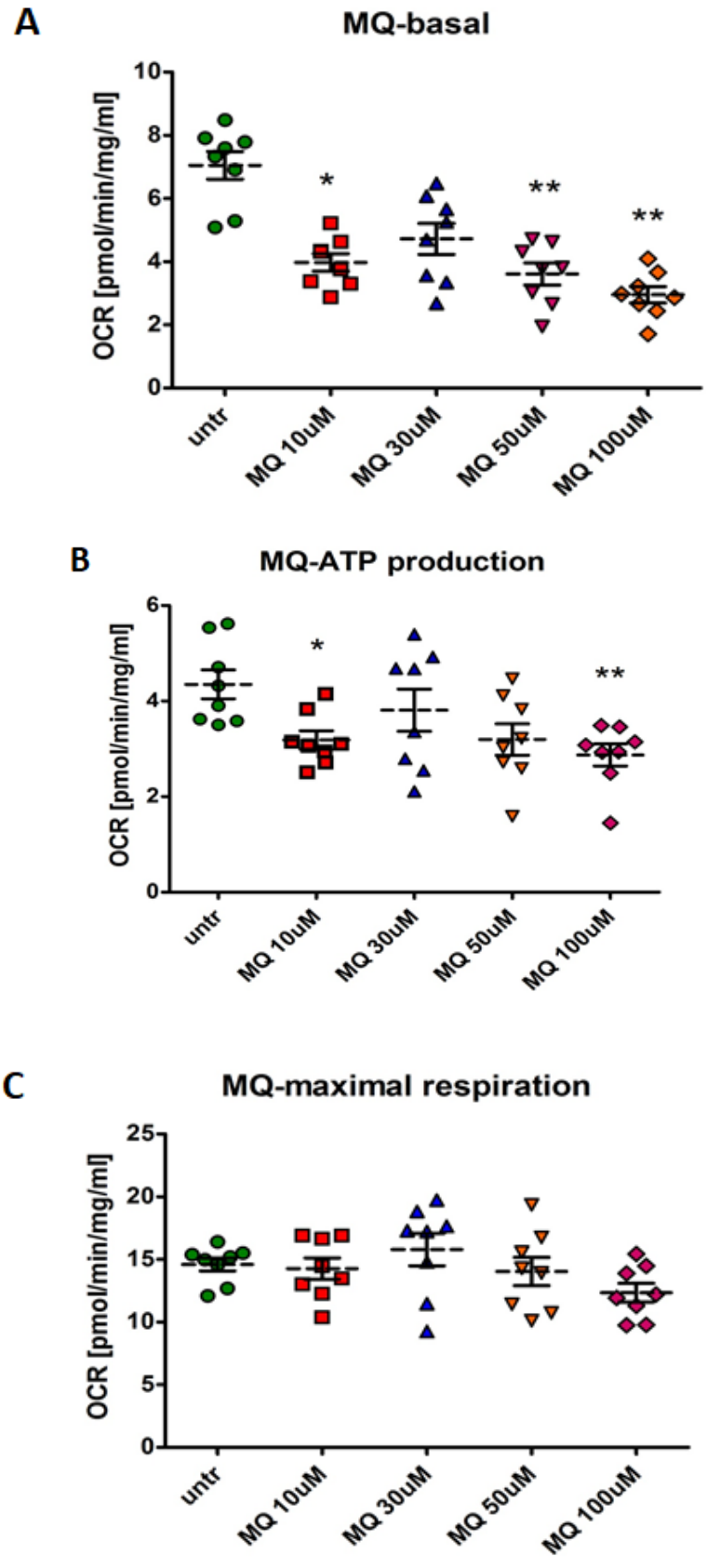


Figure 22. OCR measurement in HAECs after inhibition of NNMT by 5-Amino-1-methylquinoline. OCR profile of HAECs treated with increasing concentrations of 5-Amino-1-methylquinoline (MQ).


A
MQ-basal
OCR [pmol/min/mg/ml]
10
8
6
4
2
0
*
**
**
untr
MQ 10uM
MQ 30uM
MQ 50uM
MQ 100uM
B
MQ-ATP production
OCR [pmol/min/mg/ml]
6
4
2
0
*
**
untr
MQ 10uM
MQ 30uM
MQ 50uM
MQ 100uM
C
MQ-maximal respiration
OCR [pmol/min/mg/ml]
25
20
15
10
5
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untr
MQ 10uM
MQ 30uM
MQ 50uM
MQ 100uM

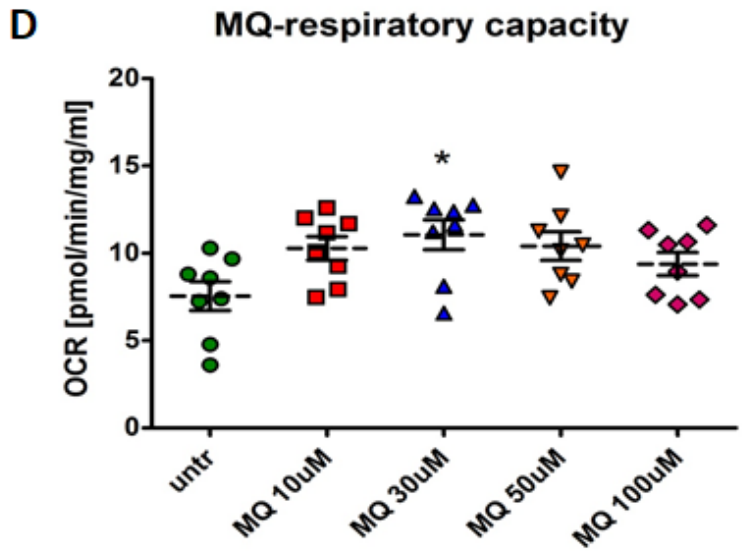


Figure 23. Seahorse XF Cell Mito Stress Test parameters in HAECs after inhibition of NNMT by 5-Amino-1-methylquinoline. Basal respiration (A), ATP production (B), maximal respiration (C), spare respiratory capacity (D) (* $p < 0.05$, ** $p < 0.01$).

3.6.2 Effect of NNMT inhibition induced by 6-Methoxynicotinamide N-methylated on HAECs mitochondrial respiration

Incubating HAECs for 1 h with increasing concentrations of 6-Methoxynicotinamide N-methylated triggered a decrease in basal respiration, ATP production, maximal respiration and spare respiratory capacity levels in a significant way (Figures 24 and 25). In this case the decrease is more pronounced than that observed for the inhibitor 5-Amino-1-methylquinoline, probably due to the ability of 6-Methoxynicotinamide N-methylated to inhibit NNMT with higher efficiency.

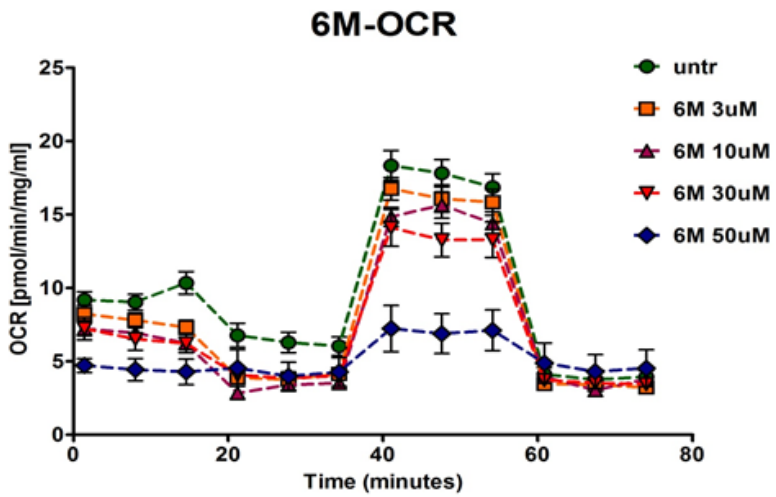
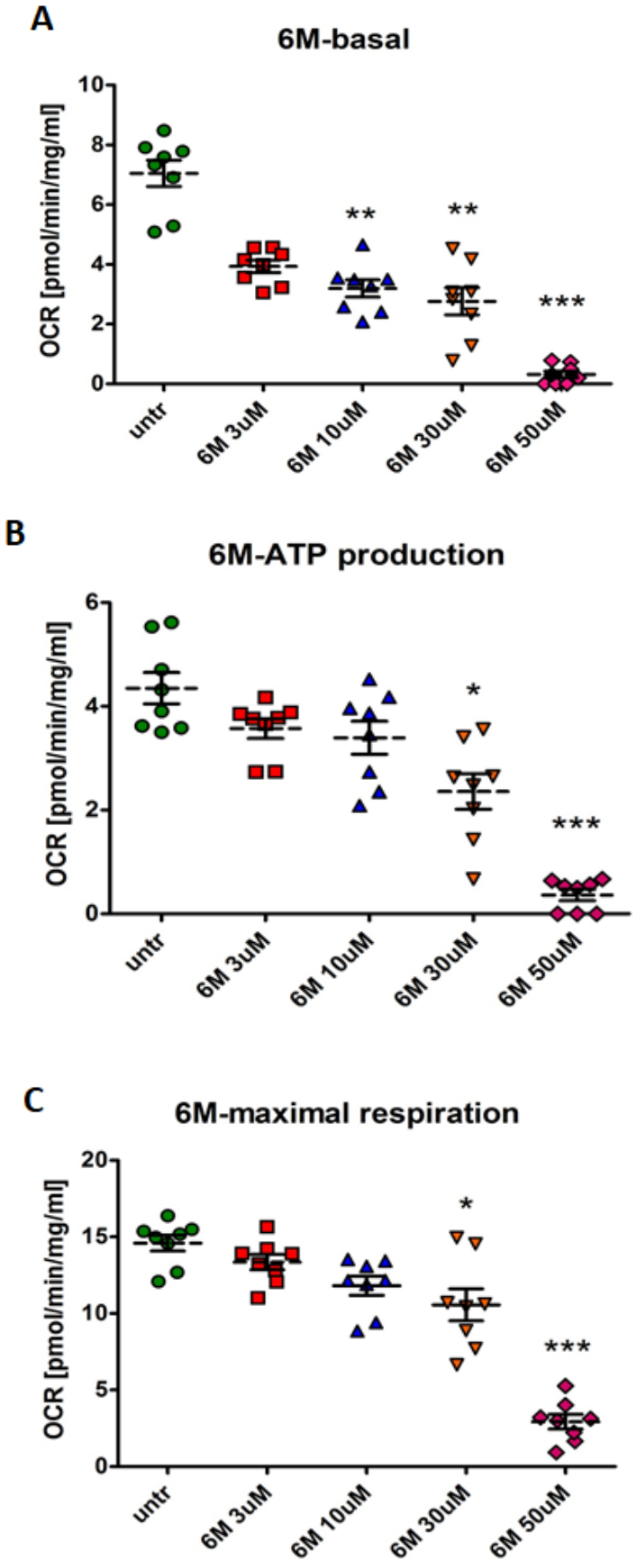


Figure 24. OCR measurement in HAECs after inhibition of NNMT by 6-Methoxynicotinamide N-methylated. OCR profile of HAECs treated with increasing concentrations of 6-Methoxynicotinamide-N-methylated (6M).


A
6M-basal
OCR [pmol/min/mg/ml]
10
8
6
4
2
0
**
**
***
untr
6M 3uM
6M 10uM
6M 30uM
6M 50uM
B
6M-ATP production
OCR [pmol/min/mg/ml]
6
4
2
0
*
***
untr
6M 3uM
6M 10uM
6M 30uM
6M 50uM
C
6M-maximal respiration
OCR [pmol/min/mg/ml]
20
15
10
5
0
*
***
untr
6M 3uM
6M 10uM
6M 30uM
6M 50uM

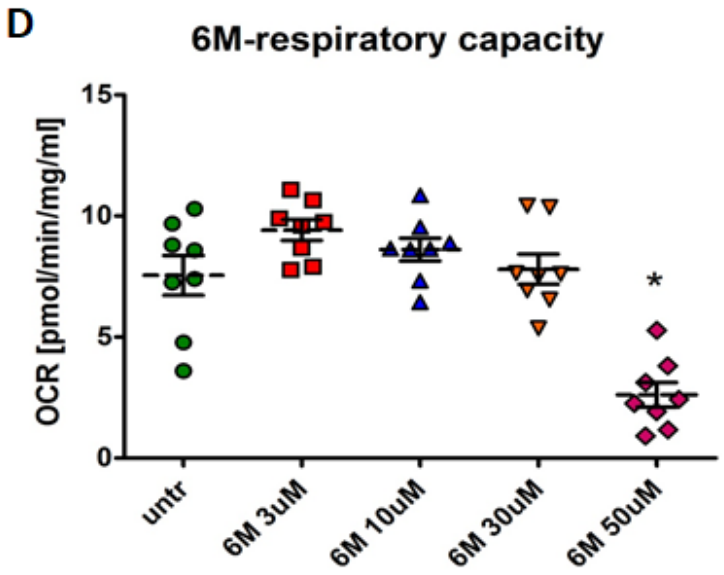


Figure 25. Seahorse XF Cell Mito Stress Test parameters in HAECs after inhibition of NNMT by 6-Methoxynicotinamide N-methylated (6M). Basal respiration (A), ATP production (B), maximal respiration (C), spare respiratory capacity (D) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.6.3 Effect of NNMT inhibition induced by 6-Methoxynicotinamide N-methylated on HAECs proliferation

Since NNMT inhibition in HAECs demonstrated to affect both mitochondrial respiration and cell viability response, it was investigated whether these variations could affect also the proliferation rate of cells.

HAECs were cultivated in full medium in the presence of 6-Methoxynicotinamide N-methylated 30 µM and at each timepoint (0, 24, 48, 72, 96 h) it was performed a MTS assay. Results were shown as ΔOD at 490 nm, obtained from the difference between the absorbance values at timepoints 24, 48, 72 and 96, and that at timepoint 0. Data obtained showed that inhibition of NNMT by 6-Methoxynicotinamide N-methylated (6MN) significantly affected proliferation of HAECs.

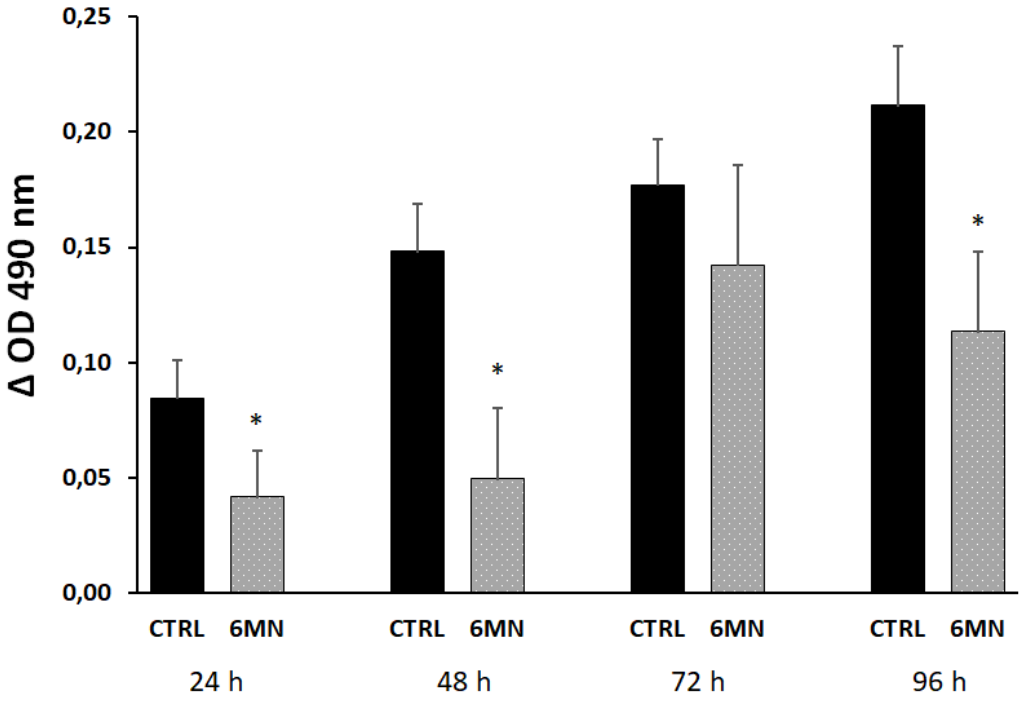


Figure 26. Proliferation rate in HAECs in which NNMT was inhibited by 6-Methoxynicotinamide N-methylated (6MN). Values are reported as mean ± standard deviation (* $p < 0.05$).

4. DISCUSSION AND CONCLUSIONS

The endothelium was considered as the internal coating of the entire circulatory system with the only functions to afford selective permeability to water and electrolytes. However, since the 80's, the endothelium was revalued as an endocrine organ that modulates complex functions. These functions include fluid filtration in the glomeruli of the kidneys, regulating blood vessel tone and blood flow, neutrophil recruitment, hormone trafficking, homeostasis with the ability to act in both sensory and effector capacities.

In the light of these observations, it is not surprising that alterations of endothelium play a crucial role in the pathogenesis of a large spectrum of serious human diseases. The endothelium is directly involved in peripheral vascular disease, stroke, heart disease, diabetes, insulin resistance, chronic kidney failure, tumor growth, metastasis, venous thrombosis, and severe viral infectious diseases.

Despite intensive research of last decades, biochemical and molecular mechanisms underlying endothelial dysfunction are still not fully understood. It was reported that NNMT, and its reaction product MNA, can potentially affect normal endothelial functions. NNMT is a phase II metabolizing enzyme, which catalyzes the N-methylation of nicotinamide, pyridines and other structural analogs, playing an important role in the biotransformation and detoxification of many xenobiotic compounds. Exogenously administered MNA showed anticoagulant [128] anti-inflammatory [130] and vascular protecting properties [134]. However, the function of endogenous MNA synthetized in endothelial cells is still unclear.

Therefore, the purpose of this work was to improve the knowledge regarding the function of NNMT in endothelium.

In order to investigate whether NNMT may have a role in the endothelial dysfunction, the cellular effects of NNMT silencing were examined in human endothelial cell line

EAhy.926. Plasmid vectors expressing shRNAs targeting different regions of NNMT mRNA sequence efficiently led to NNMT downregulation in EAhy.926.

Firstly, it was investigated whether NNMT knockdown could affect cellular metabolism in endothelial cells. Experiments of Seahorse® technology revealed very small differences in glycolysis between NNMT silenced cell lines and control, although not always significant. Subsequently, it was analyzed the mitochondrial respiration with the same technology. Surprisingly, NNMT silenced cell lines showed reduced levels of basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity compared to control line EAhy.926.

It was further investigated whether the differences observed could be a consequence of a different number of mitochondria, since it was reported that NNMT stabilizes the protein expression levels of SIRT1, a NAD-dependent deacetylase involved in mitochondrial biogenesis [106, 140]. Analysis of mitochondria number through MitoSOX™ Red staining in NNMT silenced P711 cell line and EAhy.926 showed no significant differences, suggesting that differences detected in mitochondrial respiration between silenced cells and control must be functional and not structural.

It was already reported, although in a cancer cell line, that NNMT overexpression in the human neuroblastoma cell line SH-SY5Y, which lacks of endogenous NNMT, significantly increased cell viability, Complex I activity and intracellular ATP content [101]. Results obtained in this work are consistent with these observations and suggest that NNMT can have a role, at least indirectly, in the cellular metabolism of endothelial cells too.

Given the previously reported protective properties of MNA [128, 130, 134], it was subsequently investigated whether NNMT knockdown could affect cell viability in endothelial cells. NNMT silenced cells were incubated with increasing concentrations

of menadione for 24 h and then the cell viability was evaluated by counting living cells through the fluorescent dye YO-PRO™-1 Iodide. Interestingly, data obtained showed a significant decrease of cell viability in NNMT silenced cell lines compared to mock or EAhy.926 cells transfected with empty vector. Subsequently, P711 cell line and EAhy.926 cells were subjected to two different types of oxidative stressors, by using $ONOO^-$ and H_2O_2. Cell viability was evaluated by counting living cells through the fluorescent dye YO-PRO™-1 Iodide in the case of incubation with $ONOO^-$, and by MTS when cells were incubated by H_2O_2. In both cases, NNMT silenced P711 line showed an increased sensitivity to oxidative stress-induced damage compared to control EAhy.926. While the difference in response to H_2O_2 is clearly visible especially at high concentrations of the compound (> 300 μM), $ONOO^-$ treatment showed appreciable differences only in the lowest range of concentrations used (25-75 μM) probably due to the higher reactivity of the compound.

Reactive oxygen species have been considered to play a pivotal role in endothelial dysfunction for decades [141–143].

It was reported that turnover of the SIRT1 cofactor NAD+ to nicotinamide and subsequent irreversible methylation to MNA results in generation of hydrogen peroxide by GAD-3 and a downstream mitohormetic response yielding increased stress resistance [144]. In the light of these observations, it was reasonable to expect that NNMT knockdown in EAhy.926 leads to the disruption of this delicate mechanism inducing the cells to be more sensitive to oxidative damage, and the data obtained seem to support this hypothesis.

In order to assess whether increased sensitivity to menadione injury observed in NNMT knockdown lines could be linked only to higher ROS production, it was measured the DHE fluorescence after 24 h menadione treatment. Although the DHE fluorescence

detected in NNMT silenced P711 cell line is slightly higher for the highest range of concentrations of menadione used compared to control, the increased sensitivity to menadione injury observed in NNMT silenced lines cannot be linked only to an increase in ROS production.

Therefore, it was analyzed the effect of NNMT knockdown in the P711 cell line on apoptotic/necrotic rate. Cells were treated for 24 h with peroxynitrite 30 and 50 μM and then it was performed a cytometric Annexin V/PI assay. Interestingly, data obtained showed that peroxynitrite 30 μM induced the apoptosis in the 9,2% of P711 population compared to the 0,8% of the control, and the necrosis in 80,5% of P711 population compared to the 48,5% of the control. These observations suggested that the reduced cell viability observed after NNMT knockdown upon different stressors could be linked to an increase in apoptosis, necrosis, or both.

Further, it was analyzed the cell viability after UV-C radiation. Although this measurement was performed in very basic conditions, it was observed a clear difference in the response to UV-C radiation between NNMT silenced P711 cell line and control EAhy.926.

Different stimuli have been reported to induce endothelial cell apoptosis *in vitro* (including radiation), and endothelial apoptosis has been demonstrated *in vivo* in several human diseases and animal models. Endothelial cell apoptosis can lead to disruption of the endothelial barrier with vascular leak, extravasation of plasma proteins, and exposure of a prothrombotic subendothelial matrix. Moreover, apoptotic endothelial cells are themselves procoagulant and proadhesive *in vitro*. Thus, endothelial apoptosis has the potential to be an important mechanism of vascular injury and dysfunction [145]. Vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor, angiopoietin-1 and insulin are known to provide survival

signals through engagement with their cell surface receptors VEGF-R2, FGF-R, Met, Tie-2 and IGFR respectively [146]. Upon binding, cell survival is activate through the PI3-K/Akt pathway [147]. Akt can inhibit apoptosis through multiple mechanisms and thus preventing Akt activation can induce apoptosis [148, 149]. Experiments of gene silencing, although in cancer lines, demonstrated that NNMT can affect apoptosis through the Akt pathway [103, 150]. In the light of data obtained in this work and reports present in literature, it is reasonable to believe that the same mechanism could occur in endothelial cells.

Furthermore, to confirm the results obtained with EAhy.926 cells, subsequent experiments were performed using the primary HAEC line in which NNMT activity was inhibited cultivating cells in the presence of the enzyme inhibitors 5-Amino-1-methylquinoline and 6-Methoxynicotinamide N-methylated. The efficiency of both inhibitors was tested in an experiment *ex vivo* in which aliquots of mice liver were incubated with nicotinamide 100 μM and alternatively one of the inhibitors at increasing concentrations. Thus, it was measured the MNA production at the timepoints 0, 0.5, 1, 2, 3 h through LC/MS/MS. Results showed that 6-Methoxynicotinamide N-methylated seemed more potent than 5-Amino-1-methylquinoline, since a NNMT inhibition higher than 90% was reached for a concentration of 50 μM of 5-Amino-1-methylquinoline, whereas the same efficiency was achieved with a concentration of only 10 μM for the compound 6-Methoxynicotinamide N-methylated. Subsequently, it was tested the toxicity of the compounds on HAECs. HAECs were incubated with increasing concentrations of NNMT inhibitors for 24 h and then it was assessed the cell viability by counting living cells through the fluorescent dye YO-PRO™-1 Iodide.

Both inhibitors revealed to be very safe compounds for HAECs, and no toxic effect was observed up to 5-Amino-1-methylquinoline 300 μM and 6-Methoxynicotinamide N-methylated 500 μM.

Subsequently, HAECs were incubated with increasing concentrations of menadione for 24 h and then the cell viability was evaluated by counting living cells through the fluorescent dye YO-PRO™-1 Iodide. Consistently with data obtained on NNMT silenced EAhy.926 cells, HAECs treated with NNMT inhibitor showed a significant decrease of cell viability compared to controls. This effect was clearly visible and statistical significant for HAECs treated with 6-Methoxynicotinamide N-methylated, while HAECs treated with 5-Amino-1-methylquinoline showed only a tendency, probably due to the less efficiency of this NNMT inhibitor.

Furthermore, it was investigated whether NNMT inhibition in HAECs could replicate the results previously obtained on mitochondrial respiration by NNMT knockdown in EAhy.926 cells. Experiments of Seahorse® technology revealed reduced levels of basal respiration, ATP production, maximal respiration and spare respiratory capacity in HAECs alternatively treated with NNMT inhibitors compared to control.

In the light of the data previously obtained, it was reasonable to expect differences in the proliferation rate between NNMT inhibited HAECs and control. According to our expectations, we observed a significant variation in the proliferation rate, with NNMT inhibited HAECs showing a marked decreased proliferation ability compared to control.

In many works, it was reported that NNMT enhances cell proliferation in several types of cancer, although the exact mechanism is still unclear. This work represents the first evidence in literature about a correlation between a reduced NNMT activity and cell proliferation in endothelium. Bearing in mind data obtained from this experimental work, it could be hypothesized that the mechanism responsible for the reduced

proliferation rate might be associated to a reduced mitochondrial respiration and consequently to an impaired bioenergetic balance of which effects become more evident in case of cellular stress.

In conclusion, data obtained suggest a possible involvement of NNMT in the bioenergetic regulation of endothelial cells and confirm the protective role of the enzyme in the endothelium, laying the foundations for future studies aimed to deeply understand the role of this enzyme in endothelial dysfunction. Despite NNMT being well known for its increased expression in cancer, only recently an evidence of a possible involvement of this enzyme and its reaction product MNA in endothelial dysfunction has emerged. For this reason, further studies are required to deeply understand the molecular mechanisms affected by the enzyme in endothelial cells.

5. LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ADC	Adenocarcinoma
ADP	Adenosine 5'-diphosphate
ALL	Acute lymphoblastic leukemia
ART	Mono-ADP-ribosyltransferase
ATP	Adenosine 5'-triphosphate
cADPR	Cyclic adenosine 5'-diphosphate-ribose
ccRCC	Clear cell renal cell carcinoma
CEA	Carcinoembryonic Antigen
cDNA	Complementary DNA
COMT	Catechol-O-methyltransferase
ConA	ConcanavalinA
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
GBM	Glioblastoma Multiforme
HBSS	Hank's Balanced Salt Solution
HFD	High fat diet
HNF-1β	Hepatocyte nuclear factor beta 1
HNMT	Histamine N-methyltransferase
HPLC	High-performance liquid chromatography
ICSI	Intracytoplasmic sperm injection
IL-6	Interleukin-6

LIF	Leukemia Inhibitory Factor
MNA	N1-methylnicotinamide
MPP+	1-methyl-4-phenylpyridinium ion
mRNA	Messenger RNA
MTHFR	Methylene tetrahydrofolate reductase
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NNMT	Nicotinamide N-methyltransferase
NSCLC	Non-small cell lung cancer
OSCC	Oral Squamous Cell Carcinoma
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PBS	Phosphate-buffered saline
PI	Propidium Iodide
PCR	Polymerase chain reaction
POMT	Phenol O-methyltransferase
PTC	Proximal tubular cell
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulfate

shRNA	Short hairpin RNA
Sir2	Sirtuin
SNP	Single nucleotide polymorphisms
STAT3	Signal Trasducer and Activator of transcription 3
TGF-β1	Transforming growth factor beta 1
TMT	Thiol methyltransferase
TPMT	Thiopurine methyltransferase
UC	Urothelial carcinoma

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my Supervisor Prof. Monica Emanuelli for the continuous support of my Ph.D study and related research, for believing in my abilities and giving me the opportunity to work in her laboratory, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study. I will be forever thankful to her.

Besides my advisor, I would like to give my special thanks to Dr. Davide Sartini for his important teachings, insightful comments and encouragement. He managed to teach me to work independently, but at any time, his useful advice was available to me.

I would also like to thank all my colleagues of the Laboratory of Enzymology and Clinical Molecular Biology at the Department of Clinical Sciences, Dr. Monia Cecati, Ph.D students Stefania Fumarola and Veronica Pompei. I thank them for providing a so pleasurable and friendly working atmosphere and for their always prompt help, whenever I needed it.

I would like to thank Prof. Stefan Chlopicki for hosting me at Jagiellonian Centre for Experimental Therapeutics in Krakow. It was a real privilege and a honour for me to share his exceptional scientific knowledge but also his extraordinary human qualities. This experience was really important for my professional development.

I'm also grateful to all JCET workers for making me feel at home.

I acknowledge the financial support for my research study of the European Molecular Biology Organization (EMBO Short-Term Fellowship 7274).