Hepatoblastomas exhibit marked NNMT downregulation driven by promoter DNA hypermethylation

Maria Prates Rivas1*, Talita Ferreira Marques Aguiar1*, Mariana Maschietto2, Renan B Lemes1, Luiz Carlos Caires-Júnior1, Ernesto Goulart1, Kayque Alves Telles-Silva1, Estela Novak3,4,5, Lilian Maria Cristofani3, Vicente Odone3, Monica Cypriano5, Silvia Regina Caminada de Toledo5, Dirce Maria Carraro6, Melissa Quintero Escobar7, Hana Lee8, Michael Johnston8, Cecilia Maria Lima da Costa9, Isabela Werneck da Cunha10,11, Ljubica Tasic7, Peter L Pearson12, Carla Rosenberg1, Nikolai Timchenko8 and Ana Cristina Victorino Krepischi1

Abstract
Hepatoblastomas exhibit the lowest mutational burden among pediatric tumors. We previously showed that epigenetic disruption is crucial for hepatoblastoma carcinogenesis. Our data revealed hypermethylation of nicotinamide N-methyltransferase, a highly expressed gene in adipocytes and hepatocytes. The expression pattern and the role of nicotinamide N-methyltransferase in pediatric liver tumors have not yet been explored, and this study aimed to evaluate the effect of nicotinamide N-methyltransferase hypermethylation in hepatoblastomas. We evaluated 45 hepatoblastomas and 26 non-tumoral liver samples. We examined in hepatoblastomas if the observed nicotinamide N-methyltransferase promoter hypermethylation could lead to dysregulation of expression by measuring mRNA and protein levels by real-time quantitative polymerase chain reaction, immunohistochemistry, and Western blot assays. The potential impact of nicotinamide N-methyltransferase changes was evaluated on the metabolic profile by high-resolution magic angle spinning nuclear magnetic resonance spectroscopy. Significant nicotinamide N-methyltransferase downregulation was revealed in hepatoblastomas, with two orders of magnitude lower nicotinamide N-methyltransferase expression in tumor samples and hepatoblastoma cell lines than in hepatocellular carcinoma cell lines. A specific TSS1500 CpG site (cg02094283) of

1Human Genome and Stem Cell Research Center, Department of Genetics and Evolutionary Biology, Institute of Biosciences, University of São Paulo, São Paulo, Brazil
2Research Center, Boldrini Children's Hospital, Campinas, Brazil
3Pediatric Cancer Institute (ITACI) at the Pediatric Department, São Paulo University Medical School, São Paulo, Brazil
4Molecular Genetics—São Paulo's Blood Center, São Paulo, Brazil
5Department of Pediatric, Adolescent and Child with Cancer Support Group (GRAACC), Federal University of São Paulo, São Paulo, Brazil
6International Center for Research, A.C. Camargo Cancer Center, São Paulo, Brazil
7Department of Organic Chemistry, Institute of Chemistry, University of Campinas, Campinas, Brazil
8Department of Surgery, Division of General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
9Department of Pediatric Oncology, A.C. Camargo Cancer Center, São Paulo, Brazil
10Department of Pathology, Rede D’OR São Luiz, São Paulo, Brazil
11Department of Pathology, A.C. Camargo Cancer Center, São Paulo, Brazil
*These authors contributed equally to this work.

Corresponding author: Ana Cristina Victorino Krepischi, Human Genome and Stem Cell Research Center, Department of Genetics and Evolutionary Biology, Institute of Biosciences, University of São Paulo, São Paulo 05508-090, Brazil. Email: ana.krepischi@ib.usp.br

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nicotinamide N-methyltransferase was hypermethylated in tumors, with an inverse correlation between its methylation level and nicotinamide N-methyltransferase expression. A marked global reduction of the nicotinamide N-methyltransferase protein was validated in tumors, with strong correlation between gene and protein expression. Of note, higher nicotinamide N-methyltransferase expression was statistically associated with late hepatoblastoma diagnosis, a known clinical variable of worse prognosis. In addition, untargeted metabolomics analysis detected aberrant lipid metabolism in hepatoblastomas. Data presented here showed the first evidence that nicotinamide N-methyltransferase reduction occurs in hepatoblastomas, providing further support that the nicotinamide N-methyltransferase downregulation is a wide phenomenon in liver cancer. Furthermore, this study unraveled the role of DNA methylation in the regulation of nicotinamide N-methyltransferase expression in hepatoblastomas, in addition to evaluate the potential effect of nicotinamide N-methyltransferase reduction in the metabolism of these tumors. These preliminary findings also suggested that nicotinamide N-methyltransferase level may be a potential prognostic biomarker for hepatoblastoma.

**Keywords**
Hepatoblastoma, nicotinamide N-methyltransferase, hypermethylation, epigenetics, low lipids, metabolomics

**Introduction**

Hepatoblastoma (HBL) is the most common primary liver tumor in children, accounting for \( \sim 1\% \) of pediatric cancers.\(^1\) However, it is a very rare disease with an incidence of 2.6 cases per 1 million children aged 18 years and younger.\(^2\) HBL presents a relatively normal genomic background with the lowest mutational burden reported among pediatric solid tumors.\(^3\) These embryonal hepatic tumors usually carry cytogenetic alterations, mostly aneuploidies involving gains of chromosomes 2, 8, and 20.\(^4\)–\(^6\) A few somatic mutations have already been recognized as drivers of HBL tumorigenesis, mainly as activators of the WNT pathway, with recurrent mutations in \( \text{CTNNB1} \).\(^7\)–\(^10\)

This relative paucity of molecular biomarkers poses a challenge to proper risk stratification, and gene expression signatures have been reported in recent years, providing clues about specific HBL subtypes.\(^7\)–\(^12\) A 16-gene signature discriminated two HBL subgroups that resemble early and late phases of liver development.\(^13\)

This signature stratified tumors into one group presenting fairly well-differentiated histology and favorable prognosis, and another group with a poorly differentiated histology and worse prognosis. Another model that considered differential activation of hepatic progenitor cell markers and metabolic pathways stratified HBLs in three risk groups.\(^7\) Recently, a new approach adding epigenomics to genomic/transcriptomic data resulted in an HBL risk stratification model composed of three subgroups, based on the degree of hypomethylation, as well as the expression pattern of genes located at the 14q32 locus.\(^14\)

In a previous work, we explored the role of epigenetic mechanisms in HBL by analyzing changes in DNA methylation (DNAm) in comparison to control embryonic and differentiated liver samples,\(^15\) a widespread and non-stochastic pattern of global low-level hypomethylation was disclosed in tumors, with enrichments at intergenic CpG sites. Loss of DNAm in HBL was also reported by Cui et al.\(^16\) Furthermore, aberrant DNAm in specific loci has been described in HBL samples, suggesting that epigenetic alterations are an important mechanism associated with their development.\(^17\)–\(^18\)

Our previous work evidenced that most of the detected hypermethylated sites were mapped to CpG islands,\(^15\) and a specific gene has drawn our attention due to its role in liver metabolism. The promoter region of \( \text{NNMT} \) (nicotinamide N-methyltransferase), a highly expressed gene in adipocytes and hepatocytes, was found to be hypermethylated in HBL. A previous study had shown that \( \text{NNMT} \) methylation detected in the fetal liver is lost in the differentiated liver and inversely correlated with gene expression.\(^19\) This cytosolic enzyme was initially related to the N-methylation of nicotinamide, purines, and other structural analogues\(^20\) using S-adenosyl methionine (SAM) as a methyl group donor, and the best known function of \( \text{NNMT} \) is associated with the biotransformation of drugs and xenobiotic compounds. SAM is the universal methyl donor for DNA, histones, non-histone proteins, lipids, and other metabolites, and the transfers of a reactive methyl group by NNMT to nicotinamide generate S-adenosyl homocysteine and the metabolic product 1-methylnicotinamide (1MNA).\(^21\) This activity generates a methyl sink of 1MNA, which leads to the depletion of SAM and reduces the global methylation potential of the cell.\(^22\)

To examine whether \( \text{NNMT} \) expression in HBL could be controlled by promoter hypermethylation, we determined the methylation pattern of CpG sites mapped at the promoter region and assessed the mRNA and protein \( \text{NNMT} \) expression level in tumors. Furthermore, we explored the potential impact of
**Patients and methods**

**Samples**

The samples included in this study were HBLs surgically removed between 2016 and 2019 from patients of cancer hospitals and were selected from the biobanks for presenting at least >80% of tumoral cellularity. Thirty HBL (23 fresh-frozen and 15 paraffin block—for some samples, we had material available in both conditions) samples and 11 NTCL samples were recovered from patients enrolled in three Brazilian cancer institutions: A.C. Camargo Cancer Center, GRAACC, and ITACI (São Paulo, SP, Brazil); clinical data are summarized in Table 1. The validation cohort consisted of 15 fresh-frozen HBL samples and 15 NTCL samples from the Cincinnati Children’s Hospital Medical Center in Cincinnati (USA); clinical data of this group are summarized in Table 2. Supplementary Table 1S indicates which sample was used for each experimental procedure.

The Research Ethics Committee of the respective institutions approved this research and the use of biological samples, and all samples were collected after informed signed consents from parents or children legal guardians.

Human induced pluripotent stem cell (iPSC) lines derived from the peripheral blood from two healthy individuals were obtained according to Okita et al. Using iPSCs, hepatic differentiation was performed as described by Hay et al. to obtain definitive endoderm, hepatoblasts, and precursor hepatocyte (hepatocyte-like) cells. The generation of iPSC lines derived from healthy individuals was approved by the Ethics Committee of the Institute of Biosciences, University of São Paulo, Brazil (Protocol Number 1.294.118). The liver cancer cell lines, SNU-387 (ATCC® CRL-2236™), SNU-423 (ATCC® CRL-2238™), SNU-449 (ATCC® CRL-2234™), and SNU-475 (ATCC® CRL-2236™), were acquired from ATCC (USA). Following the recommended protocol, the cell lines were grown in Roswell Park Memorial Institute Medium (RPMI 1640; Thermo Fisher, USA) supplemented with 10% Fetal Bovine Serum (FBS; ThermoFisher), in conditions at 37°C and 5% CO₂. The HBL cell lines HEPG2 (ATCC® HB-8065™) and C3A (ATCC® CRL-10741™) were grown in Minimum Essential Medium (MEM; ThermoFisher) supplemented with 10% FBS (ThermoFisher) and maintained at the same conditions.

**Gene expression**

Total RNA was isolated from HBL and NTCL samples using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer’s recommendations. Microfluidics-based electrophoresis (Bioanalyzer, Agilent Technologies, USA) was performed to verify the quality, and only RNA samples with RNA Integrity Number (RIN) >7.0 were used. Complementary DNA (cDNA) was synthesized with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) according to the standard procedures. NNMT expression was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in 35 HBL samples and 24 NTCL samples using the TaqMan® System (Applied Biosystems), and the data were normalized to the expression level of the housekeeping gene 18S ribosomal RNA (18S rRNA). All reactions were performed in triplicate in a total volume of 10 μL containing 5 μL Master Mix, 1.5 μL water, 50 ng/μL cDNA template, and 0.5 μL of the gene-specific TaqMan Assay Probe Mixture. The delta-delta Ct (ΔΔCt) method was used for data analysis, and the Kruskal–Wallis test and the post hoc Dunn test with Bonferroni correction were used for statistical analyses using GraphPad Prism 7 software.

**Protein analysis**

Qualitative protein analysis was performed in 14 available HBL samples from the Brazilian cohort by immunohistochemistry using the antibody anti-NNMT antibody (OTI3D8; Abcam, UK); reactions were automated in the BenchMark Ultra-VENTANA equipment. Images were obtained from Aperio Digital Pathology Slide Scanners - AT2 (Leica Biosystems, USA). HBL samples were classified as either positive or negative for NNMT protein expression.

Western blot assays were conducted using samples from the USA cohort. Protein extracts were isolated from available 10 HBL and 5 NTCL samples as previously described. This assay was performed in duplicate, where proteins (50 μg) were loaded onto a 4% to 20% gradient gel (BioRad, USA) and transferred to a nitrocellulose membrane (BioRad). The membranes were probed with anti-NNMT (OTI3D8; Abcam) and anti-beta-actin (ab8227; Abcam) antibodies. The results of Western blotting are also presented as ratios of the protein to the loading control, which were obtained by using ImageJ software (NIH, USA). The t-test was used in the data analysis.

**Metabolomics by nuclear magnetic resonance**

Ten Brazilian HBL samples and eight NTCL samples were available for analysis by high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy. 1H NMR spectra were acquired using a Bruker Avance spectrometer (Bruker BioSpin, Germany) operating at 400 MHz and equipped with...
Table 1. Clinical features of the 30 Brazilian HBL cases.

| ID/gender/age at diagnosis | Histology | AFP (ng/mL) | Risk stratification | PRETEXT | Chemotherapy protocol | Transplant | Metastasis | Relapse | Deceased | Other features |
|----------------------------|-----------|-------------|---------------------|---------|-----------------------|------------|------------|---------|----------|----------------|
| HB02, M, 1 m               | Epitelial and mesenchymal mixed | NA           | Low risk            | 2       | SIOPEL3               | No         | No         | No      | No       |                |
| HB05, M, 21 m              | Epitelial and mesenchymal mixed | NA           | High risk           | 4       | NA                    | Yes        | No         | No      | No       |                |
| HB15, F, 18 m              | Epitelial embryonal | 5,668,000    | Intermediate risk   | 4       | NA                    | Yes        | No         | No      | Yes      |                |
| HB16, M, 9 m               | Epitelial fetal | 824          | Intermediate risk   | 4       | SIOPEL3               | No         | No         | No      | No       |                |
| HB17, F, 36 m              | Epitelial fetal | >400,000     | Low risk            | 1       | SIOPEL3               | Yes        | No         | No      | No       |                |
| HB18, M, 9 m               | Epitelial and mesenchymal mixed | >200,000     | Low risk            | 3       | SIOPEL3               | Yes        | No         | No      | No       |                |
| HB28, M, 17 y              | Epitelial and mesenchymal mixed | NA           | High risk           | 4       | SIOPEL4               | No         | No         | Yes      | Yes      | Hepatomegaly at birth |
| HB30, M, 54 m              | Epitelial fetal with HCC features | >1,000,000   | High risk           | 2       | SIOPEL4               | Yes        | Lung       | Yes      | Yes      | Non-functional kidney |
| HB31, M, 30 m              | Epitelial fetal | 742,000      | Low risk            | 3       | NA                    | No         | No         | No      | No       |                |
| HB32, F, 36 m              | Epitelial and mesenchymal mixed | 9328000      | High risk           | 4       | SIOPEL4               | Yes        | Lung       | No      | No       |                |
| HB33, F, 1 m               | Epitelial embryonal and fetal | 28,312,000   | Intermediate risk   | 2       | SIOPEL3               | No         | No         | No      | No       | Congenital HB and unilateral renal agenesis |
| HB34, F, 19 m              | Epitelial fetal | 416,430      | Intermediate risk   | 3       | SIOPEL3               | No         | No         | No      | No       | Mother with Hepatitis C in pregnancy (no treatment) |
| HB35, M, 26 m              | Epitelial fetal | 54,800       | Intermediate risk   | 3       | SIOPEL3               | No         | No         | No      | No       | Ischemic anoxic neuropathy due to extreme prematurity |
| HB36, M, 31 m              | Epitelial embryonal and fetal | 76,348       | Low risk            | 3       | SIOPEL3               | No         | No         | No      | No       | Pilocytic astrocytoma (posterior fossa) after HB |
| HB37, F, 13 m              | Epitelial embryonal | 1,870,000    | Intermediate risk   | 2       | SIOPEL3/AHEP 0731—COG| No         | No         | No      | Yes      |                |
| HB38, F, 147 m             | Epitelial fetal | 643.4        | High risk           | 4       | SIOPEL3/AHEP 0731—COG| No         | Yes        | No      | No       |                |
| HB39, M, 84 m              | Epitelial with macrotrabecular pattern | 300,000      | High risk           | 2       | SIOPEL2               | No         | No         | No      | Yes      |                |
| HB40, M, 22 m              | Epitelial embryonal and fetal | 1842         | Low risk            | 1       | SIOPEL3               | No         | No         | No      | No       |                |
| HB42, M, 45 m              | Epitelial fetal | 1267         | Intermediate risk   | 1       | SIOPEL2               | No         | No         | No      | No       |                |
| HB43, M, 20 m              | Epitelial embryonal | 183,476      | Intermediate risk   | 4       | SIOPEL3               | Yes        | No         | No      | No       |                |
| HB44, M, 5 m               | Epitelial and mesenchymal mixed | 300,000      | Intermediate risk   | 2       | SIOPEL2               | No         | No         | No      | No       |                |
| HB45, F, 5 m               | Epitelial fetal | 445611       | Intermediate risk   | 2       | SIOPEL3               | No         | No         | No      | Yes      |                |
| HB46, M, 28 m              | Epitelial and mesenchymal mixed | >200,000     | High risk           | 4       | SIOPEL6               | No         | Lung       | No      | No       | Syndrome patient (craniosynostosis and developmental delay) |
### Table 1. Clinical Features of the 15 CCHMC HBL cases.

| ID/gender/age at diagnosis | Histology                | AFP (ng/mL) | Risk stratification | PRETEXT | Chemotherapy protocol | Transplant | Metastasis | Relapse | Deceased | Other features                      |
|----------------------------|--------------------------|-------------|---------------------|---------|-----------------------|------------|------------|--------|----------|-------------------------------------|
| HB47, M, 7 m               | Epithelial fetal        | 653,190     | NA                  | NA      | SIOPEL3               | No         | No         | No     | No       |                                     |
| HB48, M, 24 m              | Epithelial embryonal    | 60,500      | Low risk            | 2       | SIOPEL6               | No         | No         | No     | No       |                                     |
| HB49, M, 7 m               | Epithelial and mesenchymal mixed | 65,000     | High risk           | 4       | SIOPEL3               | Yes        | Yes        | No     | No       |                                     |
| HB50, M, 1 m               | Epithelial and mesenchymal mixed | NA        | Intermediate risk   | 1       | AHEP 0731—COG        | No         | No         | Yes    | Congenital HB—extreme prematurity   |
| HB72, M, 5 m               | Epithelial fetal        | 2,565,530   | Intermediate risk   | 4       | AHEP 0731—COG        | No         | No         | No     | No       |                                     |
| HB79, M, 9 m               | Epithelial fetal        | >50,000     | High risk           | 4       | SIOPEL4               | Yes        | No         | No     | No       |                                     |
| HB81, M, 20 m              | Epithelial and mesenchymal mixed | >100,000   | High risk           | 4       | SIOPEL4               | Yes        | No         | No     | No       |                                     |

**Additional Information:**
-AFP: alphafeto protein; F: female; m: months; y: years; M: male; NA: not available; HB: hepatoblastoma; HCC: hepatocellular carcinoma.

### Table 2. Continued

| ID/gender/age at diagnosis | Histology                | AFP (ng/mL) | Risk stratification | PRETEXT | Chemotherapy protocol | Transplant | Metastasis | Relapse | Deceased | Other features                      |
|----------------------------|--------------------------|-------------|---------------------|---------|-----------------------|------------|------------|--------|----------|-------------------------------------|
| 9T , F , 4 y               | Epithelial embryonal    | 304.2       | High risk           | 4       | C5VD, ICE             | No         | Yes        | Yes    | No       |                                     |
| 13T , F , 2 y              | Epithelial embryonal and fetal | 2526      | High risk           | 4       | AHEP0731, ICE, pazopanib | Yes        | Yes        | Yes    | No       |                                     |
| 14T , M, 2 y               | Epithelial fetal        | >300,000    | Intermediate risk   | 4       | C5VD                  | Yes        | No         | No     | No       |                                     |
| 16T , M, 1 y               | Epithelial with macrotrabecular pattern | 358       | Low risk            | 1       | AHEP0731              | No         | No         | No     | No       |                                     |
| 18T , F, 2 y               | Epithelial and mesenchymal mixed | 88,285     | Intermediate risk   | 3       | SIOPEL6               | No         | No         | No     | Trisomy 18                          |
| 21T , M, 3 y               | HB with HCC features    | 845,140     | High risk           | 4       | C5VD                  | No         | No         | No     | No       |                                     |
| 23T , F, 1 y               | Epithelial and mesenchymal mixed | 38,932     | High risk           | 4       | AHEP0731              | Yes        | No         | No     | No       |                                     |
| 24T , F, 3 y               | HB with HCC features    | >200,000    | High risk           | 4       | AHEP1531              | Yes        | Yes        | No     | No       | Ruptured tumor                      |
| 25T , M, 2 y               | Epithelial fetal        | >1,000,000  | High risk           | 4       | SIOPEL4               | Yes        | Yes        | No     | No       |                                     |
| 26T , F, 2 y               | Epithelial embryonal and fetal | 19,202     | Intermediate risk   | 4       | AHEP1531              | Yes        | No         | No     | No       | Extreme prematurity                 |
| 27T , F, 2 y               | Epithelial and mesenchymal mixed | 2234     | Intermediate risk   | 2       | AHEP1531              | Yes        | No         | No     | No       |                                     |
| 28T , F, 2 y               | Epithelial and mesenchymal mixed | 1179       | Intermediate risk   | 3       | AHEP0731              | No         | Yes        | No     | No       |                                     |
| 29T , M, 2 y               | Epithelial embryonal    | >300,000    | High risk           | 4       | AHEP0731, C5VD        | No         | Yes        | Yes    | No       |                                     |
| 31T , M, 2 y               | Epithelial embryonal and fetal | 7052       | High risk           | 2       | AHEP1531              | No         | Yes        | No     | No       | Trisomy 18                          |
| 33T , F, 8 y               | Epithelial embryonal and fetal | >300,000   | High risk           | 2       | AHEP1531              | No         | No         | No     | No       | Trisomy 18                          |

**Additional Information:**
-CCHMC: Cincinnati Children's Hospital Medical Center; AFP: alphafeto protein; y: years; M: male; NA: not available; HB: hepatoblastoma; HCC: hepatocellular carcinoma.
the double nuclei 4 mm probe for HR-MAS. One-dimen-
sional water-suppressed $^1$H NMR spectra were recorded
with the nuclear overhauser effect spectroscopy
(NOESY) pulse sequence and 128 repeats, and the T$_2$
edited spectra were recorded using the CPMG (Carr–
Purcell–Melboom–Gill) pulse sequence with 128 repeti-
tions. All spectra were recorded at a magic angle spin-
ning frequency of 3.5 kHz and at 293 K. Chemometrics
analysis was performed using MetaboAnalyst (www.me-
taboanalyst.ca). Details for NMR spectra processing,
data pre-processing for chemometrics, and metabolites’
assignments were previously described.30,31

**Statistical analysis**

To evaluate overall survival rates, Kaplan–Meier
method was applied by means of the survfit function
from R statistical package and the p-value from log-
rank test was reported. Nonparametric Mann–
Whitney–Wilcoxon rank-sum test was applied to inves-
tigate differences in $NNMT$ gene expression levels (RT-
quPCR) between pairs of groups. The Pearson correla-
tion coefficient ($r$) between RT-qPCR relative quantifi-
cation (RQ) values and metabolite variable importance
in projection (VIP) scores ($1^HR$ NMR HR-MAS) per
metabolite was estimated applying the Spearman non-
parametric test.

**Results**

The DNAm values of HBL and NTCL samples were
recovered from our previous work.15 These data show
that non-tumoral liver samples present a homogeneous
methylation pattern, and HBL samples present a ten-
dency of hypermethylation, although with heterogene-
ity. The HM450K platform contains 11 CpG sites
located at the $NNMT$ sequence, from which 10 reached
all quality control parameters (Supplementary Table 2;
Figure 1(a)). In Figure 1(a), boxplots of each $NNMT$
CpG site are depicted showing the level of DNAm of
HBL and NTCL samples. These boxplots and the heat-
map (Figure 1(c)) showed that the CpG sites located at
the 5' of the $NNMT$ gene are more methylated in HBL
samples than in NTCL samples. Three out of these 10 CpG
sites are located at TSS1500, but only one (cg02094283,
in green) was considered differentially methylated for
both paired and unpaired analyses.

Significant downregulation of $NNMT$ expression
was observed in the group of 20 HBL samples com-
pared to 9 NTCL samples ($p < 0.001$; Figure 2(a)).
Only four HBL samples exhibited expression levels
equivalent to NTCL samples, and two of them were
derived from HBL patients diagnosed older than
12 years. The remaining 16 HBL samples presented
$NNMT$ expression levels similar to hepatoblasts. The
analysis in iPSC samples showed a progressive increase
in $NNMT$ expression during in vitro hepatocyte differ-
etentiation; as expected, $NNTM$ expression was very low
in stem cells and derived definitive endoderm cells, while
hepatoblasts exhibited a modest increase in expression,
with hepatocyte-like cells presenting $NNMT$ expression
levels similar to NTCL samples. To confirm the reduc-
tion in the $NNMT$ expression, we used a validation
cohort of paired 15 HBL and NTCL samples, and a
decrease in the expression of $NNMT$ was also detected
in tumor samples ($p < 0.001$; Figure 2(a)). Moreover,
we found that $NNMT$ expression was two orders of
magnitude lower in the HBL cell lines HEPG2 and C3A
than in four hepatocellular carcinoma (HCC) cell lines.
The TSS1500 CpG (cg02094283) was used for the corre-
alation analysis of DNAm (M values) versus $NNMT$
expression (logged relative expression values), which
is showed in Figure 2(b); in tumors, there is an inverse cor-
relation between the methylation level of the TSS1500
cg02094283 and $NNMT$ expression ($R = –0.8271; p <
0.0001$). In addition, using data from a published
study in HBLs,7 which evaluated 50 tumor samples, we
could observe that $NNMT$ was also more expressed in
the NTCL samples, corroborating our expression find-
ings (Supplementary Figure 1S).

Qualitative $NNMT$ analysis by immunohistochem-
istry was performed in 15 Brazilian HBL. In hepatocytes
of NTCL samples, $NNMT$ protein labeling was
observed in the membrane, cytoplasm, and nucleus. A
decrease in the level of $NNMT$ protein was validated in
the majority of the HBL (Figure 3), which was detected
in several cellular components, although the loss of
protein labeling was heterogeneous in some tumors
(Supplementary Table 3S); one sample (HB50) did not
exhibit change regarding $NNMT$ protein expression.

A Western blot assay was used in the validation
cohort as another approach to verify the expression
levels of the $NNMT$ protein (Figure 4(a)). As already
revealed by $NNMT$ immunohistochemistry, the
$NNMT$ protein presented a wide reduction in HBL
samples compared to NTCL samples ($p < 0.001$;
Figure 4(b)), although its decrease was not homoge-
neous. In addition, the Pearson analysis showed a
strong correlation of the gene and protein $NNMT$
expression in tumor samples ($R = 0.93; p = 9.2e–05$;
Figure 4(c)).

Statistically significant differences on $NNMT$ gene
expression depending on HBL histological subtypes
were not detected (Supplementary Figure 2S). Using
CHIC (Children’s Hepatic tumors International
Collaboration) parameters,12,33 the HBL cases were
stratified according to their risk (Table 1). To evaluat-
ing the overall survival rates, Kaplan–Meier method
was applied considering the level of $NNMT$ expression
(Supplementary Figure 3S), considering the first
36 months after diagnosis and the level of $NNMT$
expression; the mean RQ value of the $NNMT$
Figure 1. DNA methylation (DNAm) pattern of NNMT CpG sites in hepatoblastomas (HBLs) and non-tumoral control liver (NTCL) samples. The HM450K platform contains 11 CpG sites located at the NNMT sequence, from which 10 reached all quality control parameters; DNAm beta values (ranging from 0% to 100%) of the 10 CpG sites mapped at the NNMT gene sequence were retrieved from the Infinium Illumina 450K BeadChip arrays from previous study15 in 19 HBL and 10 NTCL samples. Beta values were transformed into M values before performing comparison between groups, employing an empirical Bayesian framework linear model from limma. (a) The regions of the NNMT (TSS1500, TSS200, 5’ UTR, gene body, and 3’ UTR) are shown with the associated CpG sites depicted as numbered lollypops, according to their genomic coordinates; in green, the TSS1500 cg02094283 was differentially methylated for both paired and unpaired analyses. (b) Boxplots of each CpG site are presented showing the level of DNAm (%) of HBL and NTCL samples (median values indicated by lines); the asterisks (*) indicate the CpGs with significant differentially methylation (p < 0.03) between unpaired samples, and one of them (cg02094283) presents significant differentially methylation (p < 0.04) in both paired and unpaired analyses (**). (c) Heatmap showing the level of DNAm of the 10 NNMT CpG sites (the identification of each CpG site is given above in the columns) in group of tumors and NTCL; above is indicated the CpG site and below a DNAm level scale, in which blue and red correspond to lower and higher methylation content, respectively. The red asterisks marked the six differentially methylated CpG sites; 3 out of 10 CpG sites are located at TSS1500, and only one (cg02094283) was considered differentially methylated in both paired and unpaired analyses (data generated based on the results from Maschietto et al. 201615).
expression of all HBL samples was obtained (mean RQ = 0.15) and then HBL was classified as Group A: high expression level (above 0.15; n = 15 tumors) or Group B: low expression level (below 0.15; n = 20 tumors). No statistical difference was observed in HBL exhibiting higher or lower NNMT expression level than

**Figure 2.** Downregulation of NNMT expression in hepatoblastomas (HBLs): (a) NNMT expression analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In black dots, iPSC: induced pluripotent stem cells (iPSCs) derived from healthy individuals; DE: definitive endoderm cells; HB: hepatoblast cells; HT: hepatocyte-like cells. In blue, non-tumoral control liver (NTCL) samples from Brazilian (BR) and American (USA) cohorts. In red, tumors: HBL samples from Brazilian (BR) and American (USA) cohorts; liver cancer cell lines: in purple, HBL cell lines (HEPG2 and C3A); in gray, hepatocellular carcinoma cell lines (SNU-387, SNU-423, SNU-449, and SNU-475). For iPSC, DE, HB, and HT showing two samples for each one, corresponding to two different patients. (b) TSS1500 CpG (cg02094283) was used for the correlation analysis of DNA methylation (M values) versus NNMT expression (logged relative expression values); in tumors, there is an inverse correlation between the methylation level of the TSS1500 cg02094283 and NNMT expression (R = –0.8271; p < 0.0001).

**Figure 3.** Reduction in the expression of the NNMT protein evaluated in HBLs by immunohistochemistry. In hepatocytes, the detection of the NNMT protein in the cytoplasm and nucleus is expected (brown labeling). Panels A to E represent five HBL cases, with images obtained from Aperio Digital Pathology Slide Scanners with increases of 500, 300, and 200 μm. (a) HB31 tumor showing loss of NNMT expression in some regions. (b) HB18 exhibiting loss of expression in some regions, with complete nuclear NNMT absence in specific regions, indicated by the yellow arrow. (c) HB15 and (d) HB32 exhibiting the loss of expression in some regions. (e) HB33, a congenital tumor presenting variable NNMT expression in the epithelial-fetal region (E1), and a total absence of the NNMT labeling in the epithelial-embryonal component (E2).
the mean of the group. Following, NNMT expression level was compared between pairs of groups according to the clinical variables associated with HBL prognosis parameters (risk stratification, age at diagnosis, alpha-feto protein (AFP) dosage level in the range of 101–1000 ng/mL, PRETEXT IV, vascular invasion, metastasis, transplantation and relapse; Supplementary Figure 4Sa). It was detected a statistically significant difference related to the age at diagnosis ($p < 0.015$; Supplementary Figure 4Sb); patients diagnosed with more than 8 years old presented significantly higher NNMT expression. In addition, although not significant, the groups of patients with AFP levels 101 to 1000 ng/mL and high risk also appeared to be associated with higher NNMT expression.

We also investigated 10 Brazilian HBL samples and 8 NTCL samples using metabolomics by 1HR NMR HR-MAS, aiming to identify changes in metabolites that could be related to the reduction in the NNMT protein levels. HBL showed to be more heterogeneous than NTCL samples, as could be seen in the Partial least squares-discriminant analysis (PLS-DA) results (Figure 5(a)). The highest loadings and VIP values were observed for lipids, aromatic amino acids, and other metabolites that could be linked to NNMT lower activity in HBL (Figure 5(b) and (c)). In summary, 15 metabolites were detected with different concentrations between tumors and NTCL samples (boxplots in Figure 5(c)), 10 of them exhibiting increase in HBLs, and 5 with reduction. The five metabolites detected with reduced levels in tumors compared to NTCL samples were the amino acid tryptophan and four peaks of the triglyceride lipid class, used as an energy reserve source, both saturated and unsaturated fatty acids (–CH$_3$ (C18) cholesterol; –CH$_3$; –CH– glyceryl; –CH=CH$\equiv$). Among the 10 metabolites detected with increased concentrations, there are formate, some amino acids (tyrosine, alanine, phenylalanine), and several peaks of structural lipids, such as phospholipids, which are part of the composition of cell membranes. Typical $^1$H NMR HR-MAS spectra of HBL and NTCL samples are illustrated in Figure 5(d), where the most important metabolites (peaks 1–16) are marked.

Six HBLs and three NTCL samples were investigated using both methods $^1$HR NMR HR-MAS and RT-qPCR. Considering all studied samples, the Pearson correlation coefficients ($\rho$) between NNMT RT-qPCR RQ values and metabolite VIP scores ($^1$HR
Figure 5. Metabolomics data obtained from HBL and non-tumoral control liver (NTCL) samples: (a) Scores of the PLS-DA model obtained using T2-edited 1H NMR (CMPG) HR-MAS spectral data, constructed from data of NTCL samples (red triangles, and indicated as Group 1) and tumor (green plus sign, and indicated as Group 2). (b) The VIP values are indicated, where the colored boxes in the right show the relative concentrations of the corresponding metabolite in each group under study. (c) The boxplots show the HBL and NTCL samples levels of the top-15 altered metabolites (Student’s t-test, p < 0.05); black dots represent the concentrations of each sample, and the yellow diamond the average values of the group (HBL illustrated in green and NTCL samples in red). (d) 1H NMR HR-MAS spectrum of HBL (upper) and NTCL samples (lower): two regions are presented between 0.20 and 4.60 ppm and amplified 20 times in the regions between 5.10 and 9.00 ppm.
NMR HR-MAS) showed strong positive correlation ($\rho \geq 0.697$) in all five metabolites which are reduced in HBLs (Supplementary Table 4S and Supplementary Figure 5S), one of them statistically significant (the lipid class $-\text{CH}_3$; $p < 0.05$). In addition, all 10 metabolites with increased levels in HBLs exhibited a negative correlation with NNMT expression, although not significant.

Discussion

**NNMT** was originally identified as the enzyme responsible for the methylation of nicotinamide (NAM), producing 1MNA, which is a precursor for NAD$^+$, cofactor known for donating electrons to the mitochondrial complex I and to multiple oxidoreductases. Recent studies have expanded the role of NNMT, which has been increasingly associated with the regulation of multiple metabolic pathways in adipose and liver tissues through the consumption of methyl donor groups and the generation of active metabolites.

**NNMT** overexpression has been reported in breast cancer, glioblastoma, and papillary thyroid cancer, among others. Ulanovskaya et al. previously revealed that **NNMT** overexpression leads to an increase in 1MNA in various aggressive cancer cell lines; the authors proposed that the accumulation of 1MNA caused by **NNMT** overexpression would sequester methyl groups in cancer cells, leading to diminished methylation with changes in the epigenetic profiles.

However, it has been shown that NNMT is reduced in HCC; Kim et al. analyzed 120 patients with HCC and demonstrated that NNMT expression was reduced in the majority of the examined samples and correlated with poor prognosis. Recently, these findings were corroborated in HCC samples, with the detection of significant NNMT downregulation; in addition, NNMT expression was found to be heterogeneous, and tumors exhibiting high NNMT protein levels presented unfavorable prognostic features, such as vascular invasion and distant metastasis. Although the precise mechanisms by which the reduction in NNMT could contribute to liver cancer are not known, Shin et al. recently revealed that NNMT depletion enhances autophagy and contributes to liver cancer cell survival and tumor growth, thus providing new insights into the mechanisms of liver cancer in adult patients. Nevertheless, very little is known about the expression pattern and role of NNMT in pediatric liver cancer.

Previous work from our group identified hypermethylation at the promoter region of **NNMT** in HBL. As DNAm is a widely recognized epigenetic mechanism for the regulation of gene expression, we speculated that **NNMT** dysregulation by epigenetic changes might be a plausible factor contributing to HBL tumorigenesis, deserving further investigation. Accordingly, we examined whether DNAm alterations in HBL were correlated with **NNMT** expression. Significant **NNMT** downregulation was observed in these embryonal liver tumors compared to NTCL tissues in two different HBL cohorts. Furthermore, a specific TSS1500 CpG site (cg02094283) of **NNMT** was hypermethylated in HBL in both paired and unpaired analyses, with an inverse correlation between its methylation level and **NNMT** expression, with other neighbor CpG sites following a similar methylation pattern.

In addition, regarding the reduction in **NNMT** observed by Shin et al. in liver cancer cells, we found two orders of magnitude lower **NNMT** expression in HBL cell lines compared to HCC cell lines. In general, although the level of the NNMT protein was heterogeneous among HBL, remarkable reductions in **NNMT** mRNA and protein levels were observed using RT-qPCR, immunohistochemistry, and Western blot assays. We found a strong correlation between gene and protein expression of **NNMT** in tumor samples, indicating that the decrease in mRNA directly regulates the protein level in HBL.

In the HBL samples with available data, **NNMT** expression did not significantly impact the 36-month survival, probably because of the small sample size. However, higher **NNMT** expression was associated with known clinical variables of HBL prognosis. HBL from patients diagnosed with more than 8 years old exhibited higher **NNMT** level ($p < 0.015$), maybe because these tumors arose from more differentiated hepatocytes. It is also worthy to highlight that tumors from patients with AFP levels, 101 to 1000 ng/mL, and tumors from patients classified as high risk also appeared to be associated with higher **NNMT** expression.

Cancer cells have the ability to generate energy in a nutrient-deficient environment, and aberrant metabolism has become a characteristic hallmark of malignancies, including shifts in the metabolism of lipids. Using untargeted metabolomics by NMR, variation in many metabolites was detected in HBLs, and our data clearly showed changes in the lipid content, with an increase of the structural class of lipids, commonly detected in cancer cells because they are used for the synthesis of membranes (cytoplasmic and organelles). However, lipids used as an alternative source of energy were found to be reduced in HBLs with correlation with **NNMT** expression, suggesting that these tumors are using this class of molecules for their own energy demand for tumor progression.

Structural changes in membranes, cell signaling, gene expression, protein distribution, and disruption of energy homeostasis are caused by lipid changes, and these changes may have consequences in autophagy, necrosis, apoptosis, proliferation, differentiation, growth, and chemotherapy resistance.
Hepatocytes present the highest levels of 1MNA, evidencing a role for NNMT in the liver. Functionally, 1MNA increases SIRT1, leading to the suppression of fatty acid and cholesterol synthesis in normal hepatocytes and resulting in decreased triglyceride and cholesterol contents as well as liver inflammation. Therefore, it is expected that NNMT downregulation would impact the SIRT1 function; nevertheless, the relation between NNMT and SIRT1 is complex and modulated by several factors. Adaptations of these normal conditions were reported in cancer cells, with abnormal feedback for increased or decreased NNMT content, as well as changes in other pathways of lipid metabolism, as a source of energy. Recent studies have shown that NNMT activity consumes SAM, which decreases the methylation of selected epigenetic marks, with cell-specific effects such as fat accumulation in adipocytes. Furthermore, similar to our findings, a decrease in the lipid content in liver cells, especially affecting the polyunsaturated fatty acids, was previously reported following NNMT downregulation, providing an indirect evidence that supports the hypothesis that NNMT downregulation could diminish the lipid content in HBLs. Even though this hypothesis is appealing, the functional impact of NNMT downregulation should be evaluated in hepatocytes and their precursors to be causally associated with the detected lower lipid content in HBL.

This study provides further evidence that the reduction of NNMT in liver cancer, previously observed by others, is a wide phenomenon, now also validated in the embryonal tumor HBL. Furthermore, we have shown for the first time that HBL presented reduction in the level of specific classes of lipids. Taken together, our data highlight the role of DNAm in the regulation of NNMT expression in HBL and suggest a possible effect of NNMT depletion on the lipid metabolism.

The main findings of this study are summarized in Figure 6.

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**Author contributions**

M.P.R., T.F.M.A., M.M., and A.C.V.K. conceived the study and participated in its design. M.P.R., T.F.M.A., M.M., L.C.C.-J., E.G., K.A.T.-S., R.B.L., E.N., L.M.C., V.O., H.L., M.J., L.T., N.T., and A.C.V.K. performed the collection and assembly of data. M.P.R., T.F.M.A., M.C., R.B.L., S.R.C.d.T., D.M.C., C.M.L.d.C., I.W.d.C., M.Q.E., L.T., P.L.P., C.R., N.T., and A.C.V.K. realized the data analysis and interpretation. M.P.R., T.F.M.A., M.M., L.T., C.R., P.L.P., and A.C.V.K. wrote the manuscript. All authors have read and approved the final version of the manuscript.

**Declaration of conflicting interests**

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Guarantor

M.P.R., T.F.M.A., and A.C.V.K. are listed as guarantors of the paper.

ORCID iDs

Mariana Maschietto https://orcid.org/0000-0003-2892-3186
Estela Novak https://orcid.org/0000-0002-5732-4845
Peter L. Pearson https://orcid.org/0000-0002-5654-4264
Ana Cristina Victorino Krepischi https://orcid.org/0000-0003-2931-8605

Supplemental material

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