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Metabolomics signatures of a subset of *RET* variants according to their oncogenic risk level

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Abstract

Thirty percent of medullary thyroid carcinomas (MTCs) are related to dominant germline pathogenic variants in the *RET* proto-oncogene. According to their aggressiveness, these pathogenic variants are classified in three risk levels: 'moderate', 'high' and 'highest'. The present study compares the metabolomics profiles of five pathogenic variants, whether already classified or not. We have generated six stable murine fibroblast cell lines (NIH3T3) expressing the WT allele or variants of the human *RET* gene, with different levels of pathogenicity, including the M918V variant that is yet to be accurately classified. We carried out a targeted metabolomics study of the cell extracts with a QTRAP mass spectrometer, using the Biocrates Absolute IDQ p180 kit, which allows the quantification of 188 endogenous molecules. The data were then subjected to multivariate statistical analysis. One hundred seventy three metabolites were accurately measured. The metabolic profiles of the cells expressing the *RET* variants were found to be correlated with their oncogenic risk. In addition, the statistical model we constructed for predicting the oncogenic risk attributed a moderate risk to the M918V variant. Our results indicate that metabolomics may be useful for characterizing the pathogenicity of the *RET* gene variants and their levels of aggressiveness.

Key Words

- ▶ metabolomics
- ▶ medullary thyroid cancer
- ▶ *RET* gene
- ▶ M918V variant
- ▶ MEN2

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Introduction

Multiple endocrine neoplasia type 2 (MEN2) is a multi-glandular tumor syndrome due to germline 'gain-of-function' variants in the *REarranged during Transfection* (*RET*) proto-oncogene (Mulligan *et al.* 1994). This gene encodes a tyrosine kinase transmembrane receptor required for the proliferation and differentiation of specific neural cells, such as the progenitors of the enteric nervous system, and neuroendocrine cells, especially thyroid C cells and adrenal chromaffin cells. Given its broad roles in

tissue morphogenesis and proliferation, the *RET* gain-of-function mutations lead to MEN2 with medullary thyroid cancer (MTC), either alone or in combination with pheochromocytoma and primary hyperparathyroidism. Two distinct forms of this syndrome, MEN2A and MEN2B, are associated with distinct pathogenic variants in the *RET* gene (Wells *et al.* 2015).

Extensive study of the *RET* gene has led the American Thyroid Association (ATA) to recommend classification of

each variant according to the occurrence and aggressiveness of MTC, to go beyond the distinction of pathogenic or not pathogenic. Three classes of risk have been defined: the 'highest' risk involving the *RET* variant M918T, the 'high' risk, including variants of *RET* codons 634 and A883F, and the 'moderate' risk, including all the other variants that have been sufficiently documented (Wells *et al.* 2015). Due to the excellent genotype–phenotype correlation, this classification of prognosis is essential for defining the age at which prophylactic thyroidectomy should be performed. It also allows the selection of patients that have to be screened for pheochromocytoma, hyperparathyroidism and other symptoms caused by these *RET* variants. In the MEN2 syndrome, *RET* mutations mostly occur in exons 5, 8, 10, 11, 13, 14, 15 and 16 among the 20 exons composing the gene. Classically, MEN2A is associated with the substitution of a cysteine by another amino acid in the extracellular domain, and MEN2B, which presents a very specific phenotype, is associated with the M918T mutation in the intracellular tyrosine kinase domain.

In a recent communication, we evaluated the spectrum of *RET* mutations found in France between 2003 and 2013 (Lebeault *et al.* 2017). We found that 7.7% of the patients with MEN2 presented *RET* variants that were not classified by the ATA (Wells *et al.* 2015), a finding that compromised genetic counseling for patients and their families. To interpret the significance of these variants, we used guidelines based on various criteria such as population data, computational data, functional data and segregation data (Richards *et al.* 2015). These guidelines classify variants in five categories, that is, 'pathogenic', 'likely pathogenic', 'of uncertain significance', 'likely benign' and 'benign'. After this classification using the guidelines, 9 *RET* variants were classified as likely pathogenic, 6 as likely benign and 11 remained of uncertain significance. However, we were unable to categorize the potentially pathogenic *RET* variants unclassified by the ATA, in one of the three risk levels of the ATA recommendations based on the aggressiveness of MTC.

The mutation-related activation of the RET protein results in the major activation of cell proliferation signaling pathways, including the MAPK/ERK, PI3K/AKT, nuclear factor κ B (NF κ B), JAK/STAT and JNK pathways (Mulligan 2014). The activation of oncogenes is frequently associated with metabolic reprogramming. The common mutation V600E in the *BRAF* oncogene, found in melanoma and other cancers, induces increased mitochondrial respiration and redox stress by activating the mitochondrial pyruvate dehydrogenase enzyme (Kaplon *et al.* 2013). Yun *et al.* have shown that

cells expressing the *BRAF* or *KRAS* mutations raise the glycolytic rate by increasing the glucose uptake through overexpression of the glucose transporter GLUT1, as well as by increasing the production of lactate (Yun *et al.* 2009).

Functional studies aimed at determining the pathogenicity of *RET* variants are usually based on the ability of transfected variants to activate cell transformation and oncogenic signaling pathways. In the present study, we investigated the possibility of metabolomics signatures contributing to the classification of *RET* mutations according to their pathogenicity. We applied a quantitative targeted metabolomics approach to analyze the lipids, sugars, carnitines, amino acids and biogenic amines, in combination with assessment of the mitochondrial function, in cells expressing the WT or mutated RET protein. We then used the metabolomics signature observed at various levels of oncogenic risk to predict the risk level of the M918V variant, unclassified by the ATA. We chose to study the variant of uncertain significance (VUS) M918V since a functional study of this variant had been previously performed (Cosci *et al.* 2011), and this variant seems to have a moderate or no oncogenic level despite the fact that it occurs in the residue known to harbor the highest pathogenic mutation (M918T).

Materials and methods

Construction of the RET mutants

All *RET* mutants were obtained by site-directed mutagenesis of the *RET9* WT plasmid (p-Babe-RET9 vector, kindly provided by Dr Massimo Santoro, Napoli, Italy) using an *in vitro* mutagenesis system (Quik-Change site-directed mutagenesis, Agilent). Five missense mutations were introduced into the *RET9* plasmid: two variants associated with a 'moderate' oncogenic risk, that is, L790F and V804M; a 'high'-risk variant, that is, C634R; the 'highest' risk variant, that is, M918T; and a variant of unknown significance (VUS), that is, M918V. The WT allele was used as the control. Table 1 shows details of the variants.

Cell culture and transfection

The NIH3T3 cell line, obtained from the American Type Culture Collection (ATCC), was maintained in DMEM with F12 nutrient (DMEM-F12, PAN BIOTECH, Aidenbach, Germany) supplemented with 1% L-glutamine, 1% penicillin–streptomycin and 10% fetal bovine serum (FBS, PAN BIOTECH). Transfection was performed using Lipofectamine 2000 (Invitrogen), and the selection

Table 1 Description of the *RET* variants (transcript NCBI NM_020630.4).

ATA classification	cDNA variant	Protein variant	Short name of variant
Moderate	c.2370G>T	p.Leu790Phe	L790F
Moderate	c.2410G>A	p.Val804Met	V804M
High	c.1900T>C	p.Cys634Arg	C634R
Highest	c.2753T>C	p.Met918Thr	M918T
Non-classified (VUS)	c.2752A>G	p.Met918Val	M918V

of NIH3T3 stable clones was done using puromycin (Invitrogen).

The growth of each cell line stably transfected with the WT or mutant *RET* gene was assessed. The different stable cell lines were plated in six-well plates and treated with 100 ng/ μ L of mouse glial cell-derived neurotrophic factor (GDNF) (SRP3200, Sigma-Aldrich) during 72 h. Cell counts were made on six replicates.

Real-time PCR

Cell DNA was extracted using the EZ1 DNA extraction automate (Qiagen). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and specific primers for the *RET* gene, on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad), according to the manufacturer's instructions.

Western blot

Cells were lysed in water with protease inhibitors. The proteins, separated by SDS-PAGE in a 4–15% polyacrylamide gel (Bio-Rad), were transferred onto a PVDF membrane using the Trans-Blot Turbo system (Bio-Rad). Protein blots were probed with various primary antibodies from Cell Signaling for RET (14556S), AKT (9272S), p-AKT (13038S), 44/42 MAPK (ERK) (4695S), p-44/42 MAPK (p-ERK) (9101S) and from Abcam for α -tubulin (ab7291), diluted to 1/1000, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies, diluted to 1/10,000 and revealed by an enhanced chemiluminescence substrate (Pierce-Thermo Fischer Scientific), according to the manufacturer's instructions.

Assessment of mitochondrial respiration

The oxygen consumption rate (OCR) was measured in intact, adherent fibroblasts with an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Cells were seeded in Seahorse XF 96-well cell

culture microplates (Seahorse Bioscience) at a density of 2×10^4 cells/well in 100 μ L of culture medium and incubated for 4 h at 37°C in an atmosphere of 5% CO₂. After replacing the growth medium with 180 μ L of bicarbonate-free DMEM pre-warmed to 37°C, the cells were pre-incubated for 1 h at 37°C without CO₂ before starting the experiment. The optimal concentration of carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) required for maximal stimulation of respiration, which ranged between 2 and 14 μ M, was routinely determined by titration. After determining the basal OCR of the cells, oligomycin (1 μ M), FCCP (2–14 μ M) and antimycin A (2 μ g/mL), were sequentially added to the media and the OCR was quantified. The data were expressed as pmol of O₂ per minute, normalized by cell number.

Targeted metabolomics analysis

For metabolomics analysis, NIH3T3 cell lines were maintained in medium as described above without GDNF supplementation. When the cell culture achieved a confluence of 70–80%, adherent cells were washed with phosphate-buffered saline (PBS). Cells were then harvested using trypsin, washed and centrifuged (300 *g* for 5 min at 4°C). Metabolic quenching was performed by adding 100 μ L of a solution of cold methanol/PBS (85:15, v/v) to the cell pellet (approximately 3×10^6 cells). The mixture was transferred to a 0.5 mL homogenizer tube prefilled with 1.4 mm ceramic beads (Bertin Instruments, Montigny-le-Bretonneux, France). Cell lysis and metabolite extraction was achieved in a Precellys24 homogenizer (Bertin Instruments) by two cycles of grinding (40 s at 6500 rpm, followed by 30 s at 6000 rpm) at 4°C. The resulting homogenate was centrifuged at 10,000 *g* for 5 min at 4°C and the supernatant was stored at –80°C until analysis. Ten replicates of each stable cell line were performed with a difference of at most two cell passages between the ten samples.

We applied a targeted quantitative metabolomic approach to cell extracts using the Biocrates AbsoluteIDQ p180 kit (Biocrates Life sciences, Innsbruck, Austria). This kit, in combination with an AB Sciex QTRAP 5500 mass spectrometer (Life Sciences SCIEX, Framingham, MA, USA), allows the quantification of 188 different endogenous molecules, including acylcarnitines, amino acids, biogenic amines, glycerophospholipids, sphingolipids and sugars. After thawing on ice, 10 μ L of each sample (supernatants of the cell-lysate homogenate) were prepared according to the Biocrates Kit User Manual with ready-to-use solutions. Flow injection analysis coupled with tandem mass spectrometry (FIA-MS/MS)

was used for the analysis of carnitine, acylcarnitines, lipids and hexoses. Liquid chromatography (LC) was used for separating amino acids and biogenic amines before quantitation with mass spectrometry. After the validation of quality controls, the concentrations of metabolites were normalized to concentrations of protein cell extracts.

Statistical analysis

Multivariate data analyses were performed using the Simca P+ version 14.0 software (Umetrics, Umea, Sweden). Data were log-transformed and unit-variance scaled. We first performed unsupervised Principal Component Analysis (PCA) and so identified the similarity or the differences between sample metabolic profiles. We then used supervised analysis, the Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA), to classify samples. OPLS-DA evaluated variations between groups for each metabolite (partitioning variations that correlated with the class identifier and variations that did not contribute to discrimination between groups). The result was an OPLS-DA built from one ‘predictive’ component and two or more orthogonal components. The significance of the OPLS-DA models was assessed using the CV-ANOVA test, the Q^2 Ycum (goodness of prediction) and the R^2 Ycum (goodness of fit) values. The most relevant variables were determined from the Variable Importance for the Projection (VIP). VIP values represent the importance of these variables for the OPLS-DA models between the *Y* and *X* variables. Score plots represent the 2D projection of the two main components of models allowing the distribution and grouping of the samples, each dot representing a sample, each color a group of samples.

Univariate analysis was performed using MetaboAnalyst 3.0 (www.metaboanalyst.ca/). Mean comparisons of metabolite concentrations were performed using the non-parametric Wilcoxon test, and the Benjamini–Hochberg correction was applied to multiple tests. The results of the univariate analysis were summarized in a 2D graph known as the volcano plot with between-group metabolite fold change values on the *x*-axis and log-scaled *P* values on the *y*-axis.

Analysis of metabolic pathways

For the biological interpretation of the metabolite dataset, we mapped the quantified metabolites to the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes; www.genome.jp/kegg/), using MetaboAnalyst 3.0, a comprehensive online tool suite for metabolomics

data analysis and interpretation. This analysis was performed using the discriminant metabolites, excluding phosphatidylcholines (PCs) since their characterization cannot differentiate between the fatty acid chains.

Results

Characterization of the *in vitro* model

The copy number of the integrated *RET* gene was checked in the different NIH3T3 clones by real-time PCR to select cell lines possessing an equivalent copy number of the *RET* gene. The expression of the RET protein was verified by Western blotting (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article).

The morphology of cell lines over-expressing the mutated *RET* appeared to be modified, especially in cells carrying the RET-M918T mutation, which appeared particularly smaller (Fig. 1A). We have explored MAPK/ERK signaling and AKT signaling using Western blot. As expected, we highlighted higher activation of ERK and AKT signaling, through higher phosphorylation of these proteins, in cells expressing M918T RET variant (Fig. 1B). Cells expressing C634R and L790F RET mutations also showed higher phosphorylation of ERK and AKT proteins. WT or other RET mutations did not present relevant differences for phosphorylation of both ERK and AKT. After 72 h of treatment with the GDNF, cell growth rates were significantly higher in all RET-mutant cells compared to RET-WT cells ($P = 0.0021$ for the five mutant cells) (Fig. 1C). The rate of cell proliferation was not statistically different between the mutants, except for RET-M918T cells, which have a higher growth rate compared to other *RET* mutant cells ($P = 0.0002$) (Fig. 1C).

Cell lines overexpressing the human RET receptor showed no significant variation of mitochondrial respiration

We determined the mitochondrial respiratory rate as the mean of the cellular oxygen consumption in the different cell lines stably transfected with the *RET* gene. The basal and maximal cellular OCRs did not differ from one cell line to the other in all the cell lines studied. Mitochondrial complex III was inhibited by antimycin in order to evaluate the non-mitochondrial respiration. We observed no difference between the cell lines studied in terms of basal respiration, phosphorylating and non-phosphorylating respiration and the capacity of the electron transfer system (Supplementary Table 1).

Figure 1

(A) Cell morphology. Pictures of the cell cultures stably transfected with *RET* WT or mutant (C634R, L790F, V804M, M918V and M918T). Scale bars represent 300 μ m. (B) Cell signaling. Western blots of ERK, p-ERK, AKT and p-AKT proteins in cell lines expressing mutant or WT *RET* and the ratio of phosphorylated protein related to non-phosphorylated were calculated. (C) Cell growth. The cell growth (percentage compared to RET-WT NIH3T3 cells considered as 100%) of cell cultures stably transfected with *RET* mutants (C634R, L790F, V804M, M918V and M918T), after 72 h of GDNF treatment. All *RET* mutant cells had a significantly higher growth rate compared to WT cells (* $P < 0.01$); and RET-M918T cells had a higher growth rate compared to all the other mutant cells (* $P = 0.0002$).

Cells expressing human *RET* have distinct metabolomics signatures

Among the 188 metabolites analyzed by the Biocrates p180 kit, 173 were accurately measured in the NIH3T3 cells (Supplementary Table 2).

Applying non-supervised multivariate analysis by PCA to the six transfected cell lines and the non-transfected cell line, we highlighted a spontaneous clustering of the non-transfected NIH3T3 cell samples (NIH3T3 NT), the RET-M918T NIH3T3 and the RET-WT NIH3T3 (Fig. 2A). The other cell lines with pathogenic *RET* mutants (RET-C634R, RET-L790F, RET-V804M) and the VUS RET-M918V were not distinguished.

Discriminant analysis of these seven cell lines, using supervised multivariate analysis OPLS-DA, distinguished between the specific metabolomic signatures of the cell lines, with four clusters (Fig. 2B). Non-transfected (NIH3T3

NT) cells and RET-WT cells formed two distinct clusters, highlighting the strong influence of *RET* expression on the cell metabolomics profiles. Moreover, the model distinguished a specific metabolomics profile for mutated *RET* cells (RET-C634R, RET-L790F, RET-V804M and RET-M918V), which formed a cluster at the center of the score plot (Fig. 2B). RET-M918T cell samples formed a separate group, distinct from the other *RET* mutated cell lines, highlighting the specific metabolomics signature of these cells, which harbored the 'highest' risk *RET* mutation causing the MEN2B syndrome (Fig. 2B).

Excluding the non-transfected cells, another OPLS-DA model also showed the clustering of cells expressing the *RET* mutant and RET-WT cells (Fig. 3A). Univariate analysis of metabolomics data discriminated between these two groups. The volcano plot (i.e. fold change >2 and P value <0.05), showed a significant decrease of unsaturated, very long-chain phosphatidylcholine

Figure 2

Cells expressing human *RET* have distinct metabolomics signatures. (A) PCA plot obtained from metabolomics profiles of all the samples studied: non-transfected NIH3T3 cells (NIH3T3-NT) (grey dots), transfected with RET-WT (black dots), or mutant *RET* (C634R green dots, L790F light blue dots, V804M blue dots, M918T red dots and M918V light red dots). $R^2X = 0.841$ and $Q^2 = 0.796$. (B) OPLS-DA plot of the model performed according to the seven cell types (non-transfected or transfected with *RET* plasmids). Non-transfected NIH3T3 cells (NIH3T3-NT) (grey dots); cells transfected with RET-WT (black dots), or mutant *RET* (C634R green dots, L790F light blue dots, V804M blue dots, M918T red dots and M918V light red dots). $R^2X = 0.89$, $R^2Y = 0.849$ and $Q^2 = 0.616$. A full color version of this figure is available at <https://doi.org/10.1530/ERC-18-0314>.

(PC) species in mutant *RET* cells compared to RET-WT cells (Fig. 3B).

The metabolomics profiles of cells expressing mutated human *RET* differed according to the oncogenic risk levels

Supervised OPLS-DA analysis carried out according to risk level, with RET-L790F and RET-V804M carrying a 'moderate' risk, RET-C634R a 'high' risk and RET-M918T

the 'highest' risk, revealed a discriminant model (Fig. 4A). Using the predictive variation between X (metabolites) and Y (cells samples) variables given by $R^2X(\text{cum})$, the model interpreted approximately 87.9% of the total variation in X . This model explained approximately 95.8% of the variations in the Y vector ($R^2Y(\text{cum})$). The predictive value of the model was considered good with a $Q^2(\text{cum})$ of 0.87. The value of the CV-ANOVA test ($P = 1.92 \times 10^{-12}$) excluded the risk of over-fitting. The analysis highlighted a particularly high specific metabolic signature for the

Figure 3

Cells expressing mutant or WT *RET* have distinct metabolic profiles. (A) The OPLS-DA plot based on cells overexpressed mutant or WT *RET*. NIH3T3 cells transfected with WT *RET* (blue dots) or with mutant *RET* (green dots). $R^2X = 0.819$, $R^2Y = 0.963$ and $Q^2 = 0.839$. (B) Volcano plot of the univariate analysis of the metabolomics data of the two groups (WT and mutant *RET*), using fold change >2 and P -value <0.05 as thresholds. Graph was built with between-group metabolite fold change values on the x-axis, and log-scaled P -values on the y-axis. A full color version of this figure is available at <https://doi.org/10.1530/ERC-18-0314>.

Figure 4

The metabolomics profiles of cells expressing mutated human *RET* differ according to the risk level. (A) Score plot of the OPLS-DA model performed according to the level of oncogenic risk of *RET* mutations and obtained from the metabolomics profiles of NIH3T3 cells transfected with various mutants of *RET*: C634R as a 'high' risk mutant (green dots), L790F and V804M as 'moderate' risk mutants (blue dots), and M918T as the 'highest risk' mutant (red dots). $R^2X = 0.879$, $R^2Y = 0.958$, and $Q^2 = 0.87$. (B) Metabolic pathway analysis of discriminant metabolites according to the risk level of *RET* mutations. Metabolic pathway analyses related to the metabolites that were discriminant in the OPLS-DA of the level of oncogenic risk of *RET* mutations in cell lines using the MetaboAnalyst tools. The figure graphically summarizes the analysis of metabolite set enrichment for discriminant metabolites. The horizontal bars summarize the main metabolite sets identified in this analysis; the bars are colored according to their *P*-values, and the length is based on the fold enrichment. A full color version of this figure is available at <https://doi.org/10.1530/ERC-18-0314>.

highest risk *RET*-M918T mutated cells. The 59 metabolites supporting this discriminant model (metabolites with a VIP score >1) are shown in Table 2. Among the 59 discriminant metabolites, 19 were amino acids with relatively lower concentrations in the *RET*-M918T cells with the 'highest' risk compared to 'high'- and 'moderate'-risk cells. These differences were found significant using univariate analysis ($P < 0.0001$). Moreover, 24 of the 59 discriminant metabolites were acylcarnitines, 14 of which showed, by univariate analysis, significant differences in the 'highest' risk cells compared to 'high' and 'moderate' risk cells (Table 2). Levels of long-chain acylcarnitines were relatively lower in *RET*-M918T cells, whereas levels of short-chain acylcarnitines were relatively higher in this group.

Figure 4B shows the results of pathway analysis using the most discriminant metabolites between cells with the *RET* mutation with the 'highest' risk and those with 'high' or 'moderate' risks. Protein biosynthesis, the recycling of ammonia, the urea cycle and the metabolism of various amino acids were the most enriched pathways.

The risk level of *RET* mutation can be predicted using the metabolomics profile

We tested the prediction of the risk level for the VUS *RET*-M918V, using the OPLS-DA model generated with various *RET* mutations having a well-defined classification. A new dataset including the data of *RET*-M918V cells

was inserted in the OPLS-DA model and analyzed using the prediction tools of SIMCA software (Fig. 5). Ninety percent of *RET*-M918V cells were predicted as probably at moderate risk. The prediction of the model failed for only one of the ten *RET*-M918V samples examined.

Discussion

Metabolic alterations are considered to be emerging hallmarks of cancer as well as possible therapeutic targets (Hanahan & Weinberg 2011). We used a mass-spectrometry-based, targeted quantitative approach allowing quantification of 173 intracellular metabolites on a well-defined *in vitro* model expressing the WT or mutant *RET* oncogene. We highlighted phosphatidylcholines (PC) as a group of molecules differentially regulated between *RET*-WT and *RET* mutant cells. We also showed that the *RET*-M918T mutant cells carrying the 'highest' oncogenic risk had a specific metabolic profile characterized by highly disturbed amino acid and acyl-carnitine metabolisms.

The activation of oncogenes has been associated with the deregulation of metabolism and the proliferative capacity of cells. Indeed, in our study, *RET*-WT cells had a lower growth rate and a distinct metabolic signature compared to cells expressing the *RET* oncogene with activating mutations. Tumor growth leads to an increased turnover of membrane lipids, which play an important role in signaling and metabolic pathways.

Table 2 Metabolites with VIP scores >1 in the OPLS-DA model based on risk level groups and results of the univariate comparison (non-parametric Wilcoxon test and correction for multiple tests) of their concentrations between RET-M918T cells and other RET mutant cells.

VIP metabolites	Multivariate analysis	Univariate analysis	
	VIP score	Change in M918T vs other mutants	P value
Propionylcarnitine	1.6701	↓	0.0134
LysoPC a C20:4	1.4890	=	0.0689
Taurine	1.4256	↓	<0.0001
Octadecenoylcarnitine	1.4149	↓	0.0001
Alanine	1.4004	↓	<0.0001
Tyrosine	1.3885	↓	<0.0001
Phenylalanine	1.3844	↓	<0.0001
Isoleucine	1.3835	↓	<0.0001
Hexadecanoylcarnitine	1.3734	↓	0.0001
Arginine	1.3636	↓	0.0002
Proline	1.3608	↓	<0.0001
Leucine	1.3596	↓	<0.0001
Threonine	1.3592	↓	<0.0001
Glycine	1.3580	↓	<0.0001
Trans-4-hydroxyproline	1.3570	↓	<0.0001
Asparagine	1.3363	↓	<0.0001
Hexadecenoylcarnitine	1.3157	↓	0.0029
Methionine	1.3133	↓	<0.0001
Aspartate	1.3109	↓	<0.0001
Glutamate	1.3067	↓	<0.0001
Valine	1.3044	↓	<0.0001
Tryptophan	1.2931	↓	<0.0001
Hydroxyoctadecenoylcarnitine	1.2827	=	0.2444
Histidine	1.2824	↓	<0.0001
Ornithine	1.2574	↓	0.0001
Hydroxybutyrylcarnitine	1.2068	↑	0.0071
Methionine sulfoxide	1.2005	↓	<0.0001
Spermidine	1.1987	=	0.6801
Butyrylcarnitine	1.1953	=	0.2072
LysoPC a C14:0	1.1900	↑	0.0014
Serine	1.1724	↓	<0.0001
Citrulline	1.1696	=	0.4375
Hydroxyhexadecenoylcarnitine	1.1694	=	0.7525
LysoPC a C18:2	1.1569	=	0.7342
LysoPC a C17:0	1.1535	↓	0.0727
Propenoylcarnitine	1.1332	↑	0.0082
Octadecadienylcarnitine	1.1315	=	0.4818
LysoPC a C20:3	1.1276	=	0.7710
Hydroxypropionylcarnitine	1.1028	↑	0.0036
Octadecanoylcarnitine	1.1027	↓	0.0095
alpha-Aminoadipic acid	1.1024	↓	0.0001
Butenylcarnitine	1.1016	↑	0.0117
PC ae C30:1	1.0883	↑	0.0001
Glutaryl carnitine (hydroxyhexanoylcarnitine)	1.0785	↑	0.0011
Tiglylcarnitine	1.0702	↑	0.0349
Hexanoylcarnitine (fumaryl carnitine)	1.0660	↑	0.0071
PC aa C26:0	1.0623	↑	0.0018
Dodecenoylcarnitine	1.0546	=	0.2444
Lyso PC a C28:0	1.0497	↑	<0.0001
Tetradecenoylcarnitine	1.0485	=	0.0991
Hydroxyhexadecanoylcarnitine	1.0464	=	0.3442
Dodecanoylcarnitine	1.0455	↑	0.0257
Methylglutaryl carnitine	1.0397	↑	0.0076

(Continued)

Table 2 Continued.

VIP metabolites	Multivariate analysis	Univariate analysis	
	VIP score	Change in M918T vs other mutants	P value
Hexadecadiylecarnitine	1.0375	=	0.0991
PC ae C36:0	1.0670	↑	0.0039
Pimelylcarnitine	1.0305	↑	0.0021
PC ae C32:1	1.0203	↑	0.0007
Valerylcarnitine	1.0068	=	0.1265
LysoPC a C24:0	1.0035	↑	<0.0001

Bold *P* values indicate statistical significance.

We highlighted a decrease of levels of unsaturated, very long-chain PC species in *RET* mutant cells compared to *RET*-WT cells that could be explained by an excess of catabolism or by a synthesis defect. PCs are cleaved by a specific phospholipase C, generating diacylglycerol and phosphocholine into cytosol. In fact, increased activity of the PC-specific phospholipase C enzyme has been reported in many cancers (Glunde *et al.* 2011, Cheng *et al.* 2016). Moreover, PCs might also be an important source of second messengers capable of acting on the MAPK/ERK and PI3K/AKT signaling pathways (Glunde *et al.* 2011, Gandola *et al.* 2014). These hypotheses may explain the lipidomic profile observed in this study.

Supervised multivariate analysis also demonstrated a robust separation between the different *RET* mutants according to their oncogenic risk. Pathway enrichment analysis, using the deregulated metabolites involved in the discrimination of the three classes of *RET* mutations, highlighted a sharp deregulation of the protein biosynthesis, the recycling of ammonia, and the urea cycle. Cells expressing the *RET*-M918T mutation, that is, the mutation with the highest level of risk, showed further metabolic adaptations, probably to support an increased cell proliferation rate, increasing the consumption of many amino acids with significantly reduced

intracellular concentrations. Moreover, *RET*-M918T cells had significant variations in the levels of acylcarnitines compared to other mutant cells. Since acylcarnitines are energetic substrates for fatty acid oxidation (Reuter & Evans 2012), these variations may be due to increased mitochondrial fatty acid beta-oxidation, supporting the increased energy demands of cell growth and proliferation (Brunelli *et al.* 2016). The stable mitochondrial respiration in the presence of *RET* mutants observed in our study is consistent with this hypothesis. Deep metabolic disorders observed in *RET*-M918T cells are consistent with the very specific phenotype observed in MEN2B and reinforce the importance of distinguishing MEN2B from MEN2A.

Using the multivariate discriminant model of the three classes of risk levels, we built a predictive model for the classification of the *RET*-M918V mutant that predicted 90% of the *RET*-M918V cell lines as carrying a moderate risk of aggressive cancer. Moreover, the study of the proliferation rate of these cells highlighted a growth pattern similar to that found in cells associated with a moderate risk. These results are consistent with functional studies advocating the classification of *RET*-M918V VUS as an *RET* mutation carrying a moderate risk (Cosci *et al.* 2011). Moreover, a recent clinical study on eight patients with MTC alone and lacking the MEN2B phenotype,

Figure 5

Prediction of the risk level of NIH3T3 *RET*-M918V cells. Score plot of the prediction of the risk level of NIH3T3 *RET*-M918V cells using the OPLS-DA model for the level of oncogenic risk of *RET* mutations obtained from the metabolomics profiles of NIH3T3 cells transfected with various mutants of *RET* (Fig. 4A): C634R as a 'high' risk mutant (green dots), L790F and V804M as 'moderate' risk mutants (blue dots), M918T as the 'highest risk' mutant (red dots), and M918V as unclassified mutant (light orange dots). 90% of *RET*-M918V cells are predicted to be at moderate risk (black circle). A full color version of this figure is available at <https://doi.org/10.1530/ERC-18-0314>.

also suggested that M918V should be considered as carrying a moderate risk on the ATA classification (Martins-Costa *et al.* 2016). Our metabolomics predictive model concerning M918V variant are in agreement with previous data from the literature, suggested that metabolomics may usefully contribute to the accurate functional classification of *RET* variants and would surely be useful for the genetic counseling of patients and their families.

Limitations of the study

We have performed the study in NIH3T3 cells, a mouse fibroblast cell line which does not express *RET* receptor and commonly used as cell model for functional studies and characterizations of *RET* mutations (Colombo-Benkmann *et al.* 2008, Arlt *et al.* 2000, Borrello *et al.* 2011, Cosci *et al.* 2011, Machens *et al.* 2011, Prazeres *et al.* 2011, Colombo *et al.* 2015, Yang *et al.* 2018). However, these cells are not thyroid C cells or chromaffin cells and are not a model used to explain pathophysiological mechanism.

Expression of the *RET* receptor was not homogeneous between the cell lines, despite the fact that all transfected cells present the same copy number of the *RET* gene. Other functional studies of *RET* variants in NIH3T3 cells presented similar inhomogeneous levels of *RET* protein in their various stably transfected cell lines (Borrello *et al.* 2011, Colombo *et al.* 2015). However, the morphology and growth of the seven cell lines studied here did not correlate with the protein levels since RET-L790F had more *RET* protein than RET-V804M or RET-C634R but had the same morphology and growth level. It cannot be excluded that, besides the intrinsic activity of *RET* receptors, also their levels of expression may influence signaling (p-ERK and p-AKT) and metabolic profiles. Indeed, we noted that RET-M918T, the mutant with the most discriminant metabolic profile, is also the one expressed at the highest levels.

We used here a targeted metabolomic approach using Biocrates kit, it would be valuable to test an untargeted metabolomic approach, assessing metabolomic profiles of mutant *RET* cells for prediction of mutant pathogenicity.

Conclusion

To our knowledge, this is the first study demonstrating the efficacy of metabolomics in testing the pathogenicity of *RET* mutations. This strategy could also be used for classification of controversial *RET* variants, such as variants of the codon 791, of modifier role of some polymorphisms,

or for the evaluation of the pathogenicity of new variants discovered by high-throughput sequencing by comparing their metabolomics profiles to those of known benign or pathogenic variants.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0314>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C V-D and D M-P designed and supervised the study and wrote the manuscript; C V-D and N B performed experiments; M L M performed mitochondrial function assessment; C G and L T performed metabolomics assays; G S supervised biochemical parameters assays; C V-D performed statistical analysis; F B-C, A B and D D gave technical and intellectual support; P R gave intellectual support and conceptual advice for manuscript writing; J M C, C B, X D gave intellectual support. All authors offered conceptual advice and comments on the manuscript.

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