4138 Inhibition of the MiR-185-PAK6-Mediated Survival and Metabolic Pathways Selectively Targets Drug-Resistant CML Stem/Progenitor Cells

Andrew Wu, BSc

Overcoming drug resistance and targeting leukemic stem cells (LSCs) remain major challenges for curative treatment of human leukemia, including chronic myeloid leukemia (CML). Indeed, most patients with CML require life-long therapy with ABL1 tyrosine kinase inhibitors (TKIs), due to the persistence of residual LSCs that maintain the potential for relapse. Increasing evidence also indicates that LSCs are susceptible to cellular metabolic changes and seem to have a greater dependence on mitochondrial oxidative phosphorylation (OXPHOS) for survival. Previously, through global transcriptome profiling, we identified a key microRNA (miRNA), miR-185 as a predictive biomarker and it is also required for CML LSC survival. Its expression was significantly reduced in CD34+treatment-naïve CML cells and predictive of therapy response. Conversely, restored expression of miR-185 by lentiviral transduction in CD34+TKI-nonresponder cells significantly impaired survival of these cells, sensitizing them to TKIs in vitro and in pre-clinical xenotransplantation models, indicating that miR-185 acts as a tumor suppressor and is critical in regulating TKI response/resistance of CML stem/progenitor cells.

PAK6, a serine/threonine-protein kinase, was identified as a target gene of miR-185; it is upregulated in CD34+TKI-nonresponder cells vs. TKI-responders, correlating with reduced miR-185 expression. To further investigate the molecular and biological roles of the miR-185-PAK6 axis in the regulation of survival of drug-resistant cells, including LSCs, we performed RNA-seq and gene set enrichment analysis (GSEA) in the same CD34+patient cells where miR-185 and PAK6 were identified as being differentially expressed. Interestingly, this analysis has now identified a significant gene set enrichment of OXPHOS, reactive oxygen species (ROS), and adipogenesis pathways in CD34+CML cells compared to healthy CD34+cells (Normalized Enrichment Scores (NES): 2.44, 1.65 and 1.8). Moreover, these changes were significantly higher in TKI-nonresponder cells than in TKI-responders (NES: 1.73, 0.49 and 0.34). We have thus hypothesized that the miR-...
185-PAK6 axis may contribute to the perturbation of specific metabolic pathways in TKI-nonresponder LSC/progenitor cells and confer therapy-resistance to these cells. Indeed, a pre-clinically validated pan-PAK inhibitor (PF-3758309) alone, or in combination with a TKI, greatly reduced mitochondrial activity in TKI-nonresponder cells, in MitoTracker analysis, an effect that was not seen in the same cells treated with a TKI. Notably, ROS production was also significantly reduced in these cells treated with PF-3758309 and further reduction was observed with a combination of PF-3758309 and TKIs. Notably, PF-3758309 significantly reduced the growth of IM-resistant cell lines (IC50 25-70 nM) and CD34+TKI-nonresponder cells, as assessed by viability and colony-forming cell assays, and increased their apoptosis; these effects were greatly enhanced by TKIs (2-fold, P<0.05). These results were further confirmed in TKI-resistant cells using a lentiviral CRISPR/Cas9 knockout system that specifically targets PAK6. In addition, specific molecular changes associated with PF-3758309 treatment were also investigated using the PharmacoDB database and PharmacoGx R-package. Several candidates were identified, including growth factor independent 1B transcriptional repressor (GFI1B), a myeloid-enriched transcription factor. Its expression was reduced by PF-3758309 treatment and significantly increased in CML compared to healthy controls (>2-fold). Interestingly, expression of GFI1B is further upregulated in CD34+TKI-nonresponders compared to responders. Taken together, these findings indicate that dual targeting of miR-185-PAK6-mediated survival and metabolic pathways, along with BCR-ABL, selectively eradicates therapy-resistant LSC/progenitors, providing a valuable therapeutic strategy for improved treatment and care.

4139 NUP98-HOXA9-Initiates Molecular and Biologic Features of Disease Progression in a Humanized Model of CML

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Acute myeloid leukemias (AMLs) are heterogeneous diseases often resulting from the acquisition of multiple genetic alterations that deregulate hematopoietic precursor proliferation and block normal differentiation. Chronic myeloid leukemia offers a unique opportunity to identify molecular mechanisms that interfere with normal differentiation in the context of a highly proliferative hematopoietic stem cell clone that produces massive number of functional differentiated myeloid cells due to the presence of a BCR-ABL1 fusion gene. Since the NUP98-HOXA9 (NA9) fusion gene has been identified in some CML blast crisis patients or de novo AML, we asked whether a humanized model of CML progression to AML would result from its lentiviral-mediated introduction into primary CML CD34+ cells from 3 chronic phase CML patients in which >97%, >96%, and 44% of the CD34+ longterm culture-initiating cells were Ph+/BCR-ABL1+.

In vitro experiments showed the NA9-transduced cells produced a hugely increased number of granulo-monocytic progenitors in long-term cultures (up to 1000-fold, p=0.03; t-ratio test) and enhanced the serial replating activity of directly clonogenic cells as compared to matched samples of cells transduced with a control vector. In
vivo experiments showed that 90% of sublethally irradiated NOD-SCID IL2Rgcn-null mice expressing human IL3, GM-CSF and SCF constitutively that were transplanted with these same NA9-transduced cells developed evidence of a progressed myeloproliferative neoplasia. This included tissue infiltrates of eosinophils, basophils and mastocytes, exclusive myeloid differentiation and signs of an imminently fatal leukemia between 8 and 27 weeks post-transplant in 50% of cases, although an excess blast population was not seen in these.

RNA-sequencing of cells analyzed just 48 hours after transduction revealed a signature of 53 genes that were more highly expressed in CD34+ CML cells carrying the NA9 fusion gene in comparison to control CML cells. This signature included many genes expressed by hematopoietic stem cells (HSCs), such as 5’ HOXA genes, 3’ HOXB genes, PBX3, MEIS1, ARID5B, AHR, REL, BMP6, GDF10, SFRP5, PPBP, PLA2G4A suggesting that NA9 induces the expression of a partial HSC program in later CML progenitor types that make up the bulk of the CD34+ CML population. Consistent with this hypothesis, the NA9 signature separated the HSCs, CMPs and GMPs of chronic CML patients as well as those of normal subjects (GSE47927; p=1x10-6, p=2x10-4 respectively). Most genes in the NA9 signature were also found to be over-expressed in CD34+ cells of patients in the accelerated phase and in the blast phase of CML, including ARID5B, AHR, STARD9, TOX, or FOXP1 (p=9.10-8; ANOVA) (GSE4170). The NA9 signature was also significantly enriched in transcripts of genes that are over-expressed in the blasts of AML patients carrying NPM1 mutations or MLL fusions and was predictive of overall survival in the AML cohort of the Cancer Genome Atlas (p=0.02; log-rank test, n=200).

This stem cell signature was also associated with an increase in the number of H3K27ac marks (on average 7805 ± 960 peaks for NA9 vs 5888 ± 2739 peaks for control) at 48 hours post-transduction. De novo H3K27ac peaks in NA9+ CD34+ cells were located in proximal and distal enhancers of 993 genes (GREAT parameters TSS ± 2kb from TSS and 100kb max extension). These were significantly enriched genes in the GM-CSF signaling pathway (MySigDB, binomial p-value=2.19x10-8) and that are upregulated in granulocytes and monocytes following LPS exposure. H3K27ac peaks also matched with GATA1, RELA, MEF2A and IKZF1 transcription factor binding sites previously mapped by ChIPSeq experiments (adjusted p-value <0.05). Finally, we identified super-enhancers in 12 genes among which were PBX3, ANGPT1, MBNL1 and PRKACB.

Overexpression of the NA9 fusion gene in chronic phase CD34+ CML cells thus appears to reprogram the expression of HSC genes as well as those associated with GM-CSF pathway activation and inflammatory responses via H3K27 acetylation of associated loci leading to a picture of advanced accelerated phase/disease progression but not the complete differentiation arrest seen in terminal blast crisis or frank AML. These findings highlight the multiplicity of biologically important molecular alterations that can result from a single epigenetic perturbation but, nevertheless, are insufficient to create an overt AML phenotype.
4140 Characterization of Leukemic Stem Cells Heterogeneity in Chronic Myeloid Leukemia

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In chronic myeloid leukemia (CML), a rare subset of leukemic stem cells (LSC) persists in patients responding to conventional tyrosine kinase inhibitor (TKI) therapy. The failure to eradicate these LSCs results in indefinite therapy dependence and a risk of leukemic relapse. However, the conventional LSC compartment (Lin-CD34+CD38-) is highly heterogeneous where only a subpopulation is believed to be functional, TKI-insensitive LSCs.

Previously, using single-cell gene expression analysis we characterized the heterogeneity within the LSC population (Lin-CD34+CD38-) in CML patients using a selected panel of 96 primers. Interestingly, by comparing LSC heterogeneity at diagnosis with the heterogeneity following 3 months of TKI therapy we uncovered a therapy-insensitive, quiescent subpopulation, which could be isolated at high-purity using a combination of the surface markers: Lin-CD34+CD38-CD45RA-cKIT-CD26+ (Warfvinge, Geironson, Sommarin et al., 2017).

Here, we expand the single-cell analysis of CML LSC populations to include combined immunophenotype-/RNA sequencing analysis (CITE-seq). CITE-seq allows for unbiased, further in-depth transcriptome analysis as well as immunophenotypic characterization by pre-staining cells with a panel of DNA-barcoded antibodies prior to sequencing. DNA-barcoded antibodies convert the protein expression into readable sequences through unique oligo-conjugates as identifiers.

Using CITE-seq with a panel of 44 distinct surface markers designed to immunophenotypically differentiate between stem/progenitors cells and leukemic clones we simultaneously characterize the molecular and immunophenotypic heterogeneity within Lin-CD34+/Lin-CD34+CD38- CML stem/progenitor compartment at diagnosis. Additionally by comparing the LSCs transcriptome from patients with different therapeutic outcome after 12 months of therapy we describe how differences in heterogeneity and the presence of immunophenotypic therapy-insensitive LSCs at diagnosis (Lin-CD34+CD38-CD45RA-cKIT-CD26+) contribute to therapy response.
Leukemia emergence, maintenance, relapse and/or progression are causally linked to the presence of drug-resistant leukemia-initiating cells and impaired natural killer (NK) cell anti-tumor immune-response. Bone marrow microenvironment (BMM)-and/or leukemia-derived signals induce aberrant non-coding RNA expression and inhibit protein phosphatase 2A (PP2A) tumor suppressor activity. PP2A loss-of-function is essential for NK cell activity and leukemic but not normal stem and progenitor cell proliferation and survival. The human MIR300 gene is an intergenic miRNA that belongs to the 14q32.31 DLK1-DIO3 genomic-imprinted tumor suppressor miRNA cluster B. MIR300 was found involved in loss-of heterozygosity, inhibited in several tumor types with high mitotic index and during epithelial-to-mesenchymal transition (EMT), and associated with a cancer stem cell phenotype.

By using primary cells from Philadelphia-positive (Ph+) chronic myelogenous leukemia (CML) in chronic (CP) and blastic (BC) phase, and complex karyotype (CK) acute myeloid leukemia (AML) patients, as paradigmatic examples of stem cell-derived neoplasms characterized by constitutive expression of oncogenic kinases, PP2A loss-of-function, altered microRNA expression and impaired NK cell proliferation and cytotoxicity, we found that MIR300 is a cell context-independent tumor suppressor with anti-proliferative and PP2A-dependent pro-apoptotic activities which are sequentially activated in a MIR300 dose-dependent manner through inhibition of CCND2/CDK6 and SET (PP2A inhibitor), respectively.

To prevent PP2A-induced apoptosis, MIR300 is inhibited by oncogenic signals in CD34+CML (CP and BC) and CK-AML progenitors. Conversely, tumor-naïve BMM-induced C/EBPbeta-mediated signals (hypoxia and MSC exosomes) markedly upregulate MIR300 expression in primary CML and AML CD34+CFSEmax leukemic stem (LSC) and CD56+CD3-NK cells to induce/maintain quiescence (increased CD34+leukemic blasts in G0) and impair immune-response (suppression of NK cell proliferation and cytotoxic activity toward CD34+ leukemic blasts and CFSEmaxCD34+ CML-BC quiescent LSCs), respectively. Inhibition of MIR300 expression/activity rescues NK cell proliferation and anti-tumor cytotoxicity and prevented MSC- and hypoxia-induced growth-suppression of CD34+leukemic blasts by inhibiting degradation of MIR300 targets (e.g. SET, CCND2).

We found that CML and AML LSCs escape MIR300-induced PP2A-mediated apoptosis through the hypoxia- and tumor-dependent TGFb1-FoxM1-mediated upregulation of TUG1 lncRNA. TUG1 is an oncogenic lncRNA described as a MIR300sponge and found upregulated in solid tumors, in which it has strong
diagnostic, prognostic and therapeutic relevance and is associated with cancer stem cell maintenance and EMT.

In quiescent CML and AML LSCs, TUG1 uncouples and limits MIR300 tumor suppressor functions to cytostasis by maintaining unbound MIR300 at levels sufficient to inhibit CCND2 and CDK6 but not SET expression.

Exposure to clinically-relevant CpG-modified oligonucleotides modulating MIR300 levels and/or inhibiting TUG1 MIR300-sponging activity, restores NK cell proliferation and cytotoxic activity, and suppresses human leukemic but not normal hematopoiesis by eradicating nearly all (>95% reduction) CFSEmaxCD34+ and CD45+CD34+CD38-CD90+ LSCs and CD34+ leukemic CML (CP and BC) and CK-AML blasts in vitro (CFCs, LTC-IC, and CFSEmaxCD34+ cell tracking) and/or in NRG-SGM3 PDX mouse models of acute and chronic myeloid leukemias.

Altogether, this work highlights the therapeutic importance of altering MIR300 expression in anti-LSC and NK cell-based approaches for myeloid leukemias, and indicates that tumor-naïve BMM-induced MIR300 tumor suppressor anti-proliferative and PP2A-activating functions may support leukemogenesis by promoting the formation and initial expansion of the quiescent LSC pool through the induction of LSC dormancy and inhibition of quiescent LSC killing by cytokine-activated NK cells, respectively.

4142 Aurora Kinase a/MDM2-Mediated SETD2 Loss of Function in Chronic Myeloid Leukemia Patients in Blast Crisis Can be Therapeutically Targeted Inducing Apoptotic Cell Death in a Caspase-Dependent Way

Manuela Mancini, PhD

One of the hallmarks of chronic myeloid leukemia (CML) is genomic instability, that fosters the acquisition of tyrosine kinase inhibitor (TKI)-resistant BCR-ABL1 mutations and/or of additional chromosomal aberrations leading to progression to blast crisis (BC).

Inactivating mutations in the SETD2 tumor suppressor occur in solid tumors and acute leukemias. SETD2 trimethylates histone H3 Lysine 36 (H3K36Me3) playing a key role in maintaining DNA integrity. We have recently demonstrated that, in CML, SETD2 loss of function may occur at the post-translational level. Reduced or null SETD2 and H3K36Me3 was detected in 83/96 (86%) patients (pts) with BC CML as compared to a pool of healthy donors and to chronic phase (CP) pts at diagnosis. Proteasome inhibition in primary cells from pts with undetectable SETD2 restored H3K36Me3 and led to accumulation of hyper-ubiquitinated SETD2. In K562 cells (SETD2/H3K36Me3low), we observed that after proteasome inhibition
hyper-ubiquitinated SETD2 co-immunoprecipitates with MDM2. MDM2 inhibition rescued SETD2 expression and activity, suggesting that MDM2 is implicated in SETD2 reduced stability. Co-IP also showed that SETD2 interacts with Aurora Kinase A (AKA) a S/T kinase frequently overexpressed in CML. We found that AKA phosphorylates SETD2, and its inhibition rescued SETD2 expression and activity.

To investigate whether SETD2/H3K36Me3 loss may be a druggable lesion, we performed clonogenic assays in LAMA84 (SETD2/H3K36Me3high) cells before and after SETD2 silencing, in imatinib-sensitive K562 (SETD2/H3K36Me3low) cells and in IM-resistant K562 cells, that are characterized by complete SETD2 loss. The extent of reduction of clonogenic growth after proteasomal, AKA or MDM2 inhibition was found to be inversely correlated to SETD2 residual expression. These observations were confirmed in cells from both CP (n=2) and BC (n=4) CML pts showing different levels of SETD2 expression and activity.

Further experiments were performed in the aforementioned cell lines to verify if reduced clonogenic potential was due to cytostatic or cytotoxic effects. Apoptotic cell death was quantified by annexin V/propidium iodide staining and flow cytometry. Proteasomal inhibition by bortezomib, carfilzomib and ixazomib and AKA de-phosphorylation by Danusertib caused a time-dependent increase of annexin-V-positive cells by activating the mitochondrial apoptotic pathway as reflected by an increase in Bax expression and induction of the cleavage of caspase-3,-9 and PARP. Moreover, all drug treatments as single agent, at nanomolar doses (Bortezomib: 10 nM, Carfilzomib: 5 nM, Ixazomib: 10 nM and Danusertib: 500 nM) induced a significant increase of the DNA double-strand break marker γH2AX, suggesting that in a SETD2 knock-down context, proteasomal and AKA inhibition propagates genomic instability by forcing the cells through successive replication cycles, ultimately resulting in apoptosis from mitotic catastrophe.

Reduced SETD2/H3K36Me3 levels, in association with MDM2 and AKA hyper-activation, were also detected when the CD34+ cell fraction of 10 CML-CP pts, was compared to the total mononuclear cell fraction or to the CD34+ compartment obtained from a pool of healthy donors. We thus hypothesized that leukemia progenitor cells, showing higher MDM2 and AKA activity and consequent SETD2 loss, accumulate genetic aberrations despite inhibition of BCR-ABL1 kinase. Studies are ongoing to verify if MDM2 or AKA inhibition may restore SETD2 expression and function and induce cell death.

Finally, it has already been shown that alterations of epigenetic regulators such as the KDM4 family members control tumor cell proliferation in a variety of cancers including acute myeloid leukemia. Recent findings have identified KDM4 demethylases as putative therapeutic targets in a SETD2 mutated context and illustrated the efficacy of KDM4 inhibitors in AML therapy. Starting from these evidences, we will test the same approach in BC CML models.
In conclusion, phosphorylation by AKA and ubiquitination by MDM2 contribute to SETD2 non-genomic loss of function in BC CML and in CD34+ leukemic progenitors. Restoring physiological H3K36Me3 may help to improve the outcome of this critical subset of pts.

4143 Simultaneous Inhibition of BCR-ABL1 Tyrosine Kinase and PAK1/2 Serine/Threonine Kinases Exerts Synergistic Effect Against Chronic Myeloid Leukemia Cells

Sylwia Flis, PhD, DSc

Tyrosine kinase inhibitors (TKIs) revolutionized the treatment of BCR-ABL1 tyrosine kinase - positive chronic myeloid leukemia in chronic phase (CML-CP). However, it is unlikely that TKIs will “cure” the disease in majority of patients because CML-CP cells are elusive targets even for the most advanced therapies employing second and third generation of TKIs. Therefore, new treatment modalities are necessary to improve therapeutic outcomes.

We showed before that class I p21-activated serine/threonine kinases (PAKs) remained active in TKI-naive and TKI-treated CML-CP leukemia stem and early progenitor cells. The aim of the study was to test whether simultaneous inhibition of signaling pathways activated by BCR-ABL1 and PAK kinases may improve the treatment of CML. Special attention was focused on PAK1 and PAK2, which are expressed in hematopoietic cells and can play an important role in the promotion of CML cells proliferation and survival. PAK kinases were targeted by small molecule inhibitor IPA-3 (inhibitor of PAK1) and shRNA construct for PAK2, BCR-ABL1 kinase was inhibited by imatinib. The studies were carried out using (i) primary CML-CP stem/early progenitor cells and normal hematopoietic counterparts isolated from the bone marrow of newly diagnosed CML-CP patients and healthy donors, respectively, (ii) CML-blast phase cell lines (K562 and KCL-22), and (iii) BCR-ABL1-transformed 32Dcl3 cell line cells.

We show here that inhibition of PAK1 or/and PAK2 kinases activity enhanced the effect of IM against CML cells without affecting normal counterparts. We observed that the combined use of IM with IPA-3 increased growth inhibition and apoptosis of leukemia cells. To evaluate the type of drugs interaction median effect analysis method was used. The studies revealed that the type and strength of drug interaction depend on drug concentration. Generally, combination of IM with IPA-3 at the 50% of the cell kill level (EC50) generated synergistic effect. Altogether, we postulate that BCR-ABL1 kinase inhibitor should be combined with PAK1/2 inhibitor to facilitate eradication of CML cells.
4144 Metabolomics Profile of Patients with Chronic Myeloid Leukemia and Cardiovascular Adverse Events after Treatment with Tyrosine Kinase Inhibitors

Giovanni Caocci, MD

**Background.** Cardiovascular adverse events (CV-AE) are emerging complications in chronic myeloid leukemia (CML) patients treated with second and third generation tyrosine kinase inhibitors (TKIs). Despite the importance of CV risk factors, predictive CV-AE biomarkers are still lacking. Further understanding of the molecular pathways underlying CV-AE may promote novel strategies to prevent its initiation prior to clinical disease. In this scenario, the use of a novel tool such as metabolomics may be useful for the identification of new metabolic pathways related to CV-AE. Metabolites are the output of cellular metabolism, accounting for expression and activity of genes, transcripts, and proteins, and offering unique insights into small molecule regulation. For the first time we evaluated the correlation between CV-AE and metabolomic profile in CML patients treated with TKIs.

**Methods.** We considered 39 adult CP-CML patients (mean age 49, range 24-70), without comorbidity at baseline, consecutively diagnosed and treated with imatinib, dasatinib nilotinib and ponatinib, at the Haematology Unit of “Businco Hospital”, Cagliari, Italy. All patients underwent a metabolomic profile detection, after CV-AE or during follow-up, and were stratified in 2 groups (with or without CV-AE). Plasma samples were collected and acquired chromatogram was analysed by means of the free software AMDIS (Automated Mass Spectral Deconvolution and Identification System; http://chemdata.nist.gov/mass-spc/amdis) that identified each peak by comparison of the relative mass spectra and the retention times with those stored in an in-house made library comprising 255 metabolites. Data were investigated by applying the supervised multivariate statistical approach OPLS-DA (Orthogonal partial least square discriminant analysis) (SIMCA, version 13.0, Umetrics, Umea, Sweden).

**Results.** The mean follow-up since CML diagnosis was 3.7 years (range 0.9-5); 22 (56.4%) patients were treated frontline, while 17 (43.5%) underwent second or subsequent TKI lines of treatments. The reason for switching was inefficacy in 15.3% and intolerance in 28.2%. At CV-AE or last follow-up 16 (41%) patients were treated with imatinib, 8 (20.5%) with dasatinib, 14 (35.8%) with nilotinib and 1 patient with ponatinib (2.7%). Overall, 17 CV-AE were recorded: 7 cases of hypercholesterolemia, 5 pleural or pericardial effusions, one episode of hypertension and 4 cardiac events (atrial fibrillation,ST-segment elevation myocardial infarction, reduction of cardiac ejection fraction and dissecting aneurysm of the aorta); 7 CV-AE were graded as 3 according to the common toxicity criteria and one patient died from dissecting aneurysm of the aorta). The 60-month cumulative CV-AE incidence was 54.4±9.1%. The mean time between the start of the treatment and the occurrence of a CV-AE was 44.4 months (range 19-60). OPLS-DA showed that
patient’s samples were clearly separated into 2 groups indicating that CV-AE patients (blue dots) presented a markedly distinct metabolic profile compared with patients without CV-AE (green dots); (figure 1). The parameters of the model were $R^2Y = 0.76$ and $Q^2 = 0.44$. To validate the OPLS-DA model, a permutation was performed resulting statistically significant ($p=0.002$). The main discriminant metabolites were tyrosine, lysine, ornithine, glutamic acid, 2-piperidincarboxylic acid, proline, citric acid, phenylalanine, mannitol, threonine, leucine, creatine, serine, 4-hydroxyproline, and alanine (more represented in CV-AE group); while unknown 204, myristic acid, arabitol, oxalic acid, 4-deoxyrithronic acid, elaidic acid and ribose resulted less expressed in CV-AE group.

**Conclusions.** This exploratory study showed different metabolomic profile of CML patients with CV-AE underwent TKI treatment, suggesting possible mechanisms of endothelial damage mediated by the accumulation of metabolites. Tyrosine, highly expressed in the CV-AE CML group, is a reliable marker of oxidative stress in various acute and chronic diseases. Metabolomics research has considerable potential for translating the metabolic fingerprint into personalized therapeutic strategies. These preliminary data should be confirmed in prospective clinical trials.