EARLY ANTIRETROVIRAL THERAPY INITIATION EFFECT ON METABOLIC PROFILE IN VERTICALLY HIV-1-INFECTED CHILDREN

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Synopsis

Background: Early combined antiretroviral treatment (cART) in perinatally acquired HIV-1-children has been associated with a rapid viral suppression, small HIV reservoir size and reduced children mortality and morbidity. Immunometabolism has emerged as an important field in HIV-1 infection offering both relevant knowledge regarding immunopathogenesis and potential targets for therapies against HIV-1.

Objective: To characterize the proteomic, lipidomic and metabolomic profile of HIV-1-infected children depending on their age at cART initiation.

Patients and methods: Plasma samples from perinatally HIV-1-infected children under suppressive cART who initiated an early cART (first 12 weeks after birth, EARLY, n=10) and late cART (12-50 weeks after birth, LATE, n=10) were analysed. Comparative plasma proteomics, lipidomics and metabolomics analyses were performed by nanoLC-Orbitrap, UHPLC-qTOF and GC-qTOF, respectively.

Results: Seven of the 188 proteins identified exhibit differences comparing EARLY and LATE groups of HIV-1 infected children. Despite no differences in the lipidomic (n=115) and metabolomic (n=81) profiles, strong correlations were found between proteins and lipid levels as well as metabolites, including glucidic components and amino acids, with clinical parameters. The ratio among different proteins showed high discriminatory power of EARLY and LATE groups.

Conclusions: Protein signature show a different proinflammatory state associated with a late cART introduction. Its associations with lipid levels and the relations found between metabolites and clinical parameters may potentially trigger premature non-AIDS events in this HIV population including atherosclerotic diseases and metabolic

disorders. Antiretroviral treatment should be started as soon as possible in perinatally acquired HIV-1-infected children to prevent them from future long-life complications.

Introduction

Each year, more than 180,000 infants still become infected via mother-to-child transmission (MTCT) of HIV despite the fact that the implementation of guidelines for prevention with combined antiretroviral treatments (cART) has greatly reduced the incidence of perinatal HIV transmission. [1]Timing of early treatment has been widely discussed and varied in multiple clinical studies spanning from only few hours after birth [2] up to one year of life.[3] However, what it has currently been well established is that, regardless of clinical staging of CD4 cell count, the earlier the treatment is initiated after infection, the greater the benefits are for HIV-infected children.[4,5] A better understanding of the clinical, immunological and virological effects induced by early cART initiation could be relevant for developing treatment interventions for neonates with HIV-1 infection.

Evidences from clinical trials and observational studies indicate that early cART initiation reduce children morbidity and mortality.[6–8] Furthermore, we and others showed that early cART initiation brings benefits such as a rapid viral suppression and small HIV-1 reservoir size, preventing the HIV-1 DNA integration in the peripheral blood cells and other tissues.[2,9–13] Several immunological factors have been associated with a small reservoir size after early cART including a preserved memory B-cell response and HIV-1-specific CD4 and CD8-T cell response, reduced T-cell immune activation and a distinct innate immune profile with improved antiviral activity.[14–16] Limitations on the establishment of reservoirs caused by early cART initiation may play an important role in achieving viral natural control upon cART interruption, which is called “functional cure”.[2,17,18] Therefore, the process of how very early cART affects persistence of HIV-1 children with suppressed viraemia should be well understood.

Immunometabolism, study of the interplay between bioenergetics pathways and immune cell functions, has emerged as an important field in HIV-infection. Activation of the host innate and adaptive immune cells that characterized chronic infections are accompanied by dramatic changes in cellular bioenergetics [19,20] and a specific proteomics and metabolomics profile has been associated with persistent spontaneous HIV control in the absence of cART.[21,22] Proteomics has associated the loss of virological control to inflammation, transendothelial migration and coagulation. Interestingly, cellular metabolism has recently been considered as a major determinant of HIV-1 reservoir on CD4-T cells, more strongly than the state of cell differentiation and/or activation. In fact, CD4-T cells with higher metabolic activity levels (high oxidative phosphorylation and glycolysis) are more susceptible to HIV-infection,[23] highlighting the cellular metabolism as an important target for new therapies against HIV-1 infection.

The majority of findings related to immunometabolism in HIV-infection obtained so far have been focused on adults or on *in vitro* assays and there is scarce information on the metabolic profile in HIV-infected children.[24] Based on the link between the cellular metabolic requirements for reservoir establishment on CD4-T cells and our previous results in which an early cART initiation was associated with low viral reservoir,[10] we conducted this cross-sectional study to determine if this group of early treated perinatally acquired HIV-1-children can be also characterized by a distinctive metabolic profile. All samples were obtained before a short time of cART initiation due to not samples availability before cART initiation.

Patients and Methods

Study Participants

This retrospective study of a prospectively collected cohort is based on children with vertically transmitted HIV infection from the Paediatric Spanish AIDS Research Network Cohort (coRISpe). All participants were Caucasian. Twenty patients, all of them on suppressive cART treatment at sample recruitment, were included based on the plasma sample availability and the following inclusion criteria: 1) only samples from those children who initiated cART within the first year of life were included; 2) children had evidence of virological suppression within the first year after cART initiation; 3) had subsequent maintenance of viral control (≤20 copies/mL) for at least six months before sampling; and 4) not coinfected by Hepatitis C Virus (HCV). Cryopreserved plasma and associated clinical data were provided by the Spanish HIV HGU BioBank [25] and by coRISpe.[25] Patients were classified in two groups according the initiation of cART in those who initiated cART early (EARLY, 0-12 weeks, n=10) and those who initiated cART late (LATE, 12-50 weeks, n=10).

The study was approved by the ethic committee of Hospital General Universitario Gregorio Marañón (HGUGM) in Madrid.

Proteomics analysis

Comparative plasma shotgun proteomics was performed with tandem mass tag (TMT) isobaric tag labelling and nanoflow liquid chromatography coupled to Orbitrap mass spectrometry for no-hypothesis driven proteomic studies. Detailed information about protein extraction and identification can be found in Supplementary Material.

Protein identification and quantification were performed on Proteome Discoverer software v.1.4.0.288 (Thermo Fisher) by Multidimensional Protein Identification Technology (MudPIT) combining the three raw data files from each TMT plex. For protein identification, all MS and MS/MS spectra were analysed using Mascot search engine (v.2.5) combing Homo Sapiens (74449 entries) and contaminants (247 entries) databases. Two missed cleavages were allowed and an error of 0.02 Da for FT-MS/MS fragmentation mass and 10.0 ppm for a FT-MS parent ion mass were allowed. TMT-10plex was set as quantification modification and oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modifications. The false discovery rate (FDR) and protein probabilities were calculated by Percolator. For protein quantification, the ratios between each TMT-label against 126-TMT label were used and quantification results were normalized based on protein median.[23]

Metabolomics analysis

Samples were analysed on a 7200 GC-QTOF Gas chromatograph-quadrupole time of flight mass spectrometer (GC-qTOF) from Agilent Technologies (Sta. Clara, CA, USA).[19,21]

Absolute quantification of the Krebs cycle related metabolites was performed with an internal standard calibration curve using the corresponding analytical standard for each metabolite.[27] An untargeted approach was performed by deconvoluting the acquired raw data by Unknown Analysis software from Agilent and using Fiehn RT library to identify, by EI-MS spectra and library retention time, other metabolites. These compounds were semi-quantified in terms of internal standard response ratio.

Lipidomic analysis

Lipid extraction procedure can be found in supplementary material. Samples were analysed on an Agilent 1290 Infinity UHPLC coupled to an Agilent 6550 qTOF mass spectrometer. The chromatographic separation consists of an elution with a ternary mobile phase containing water, methanol and 2-propanol with 10mM ammonium formate and 0.1% formic acid. The stationary phase was a C18 column (Kinetex EVO C18 Column, 2.6 µm, 2.1 mm x 100 mm) that allows the sequential elution of the more hydrophobic lipids such as lysophospholipids, sphingomyelins, phospholipids, diglycerides, triglycerides and cholesteryl esters, among others. The identification of lipid species was performed by matching their accurate mass and tandem mass spectrum, when available, to Metlin-PCDL from Agilent containing more than 40,000 metabolites and lipids. Chromatographic behaviour of pure standards for each family and bibliographic information was used to ensure their putative identification. After putative identification of lipids, these were semi-quantified in terms of internal standard response ratio using one internal standard for each lipid family.[21]

Statistical analysis

Variables were considered as non-parametric due to the sample size. Differences between categorical and continuous values were determined by the Chi-square test and Mann-Whitney U test respectively. Correlations between variables were assessed using the Spearman rank test. p values <0.05 were considered statistically significant. Receiver operating characteristic curves (ROC) were built to quantify how accurately proteins, metabolites and lipids were able to discriminate between the groups. Information regarding normal protein function was obtained from the UniProt database and the protein-protein interaction network was generated using the STRING database, version 11.[28] FDR was used to evaluate how significant the enrichment is and p-values corrected for multiple testing within each category using the Benjamini–Hochberg

procedure. Representative nanoLC-(Orbitarp)MS/MS chromatograms for omics studies are shown in Figure S1. The statistical software used included the programme “R”, v3.4.4 (http://cran.r-proyect.org/) and the SPSS 23.0 package (IBM, Madrid, Spain). Graphs were generated with Prism, v9.0 (GraphPad Software, Inc.).

Results

General and clinical characteristics of the studied subjects

Clinical and demographic characteristics of studied subjects are shown in **Table 1**. Comparing both groups, statistical differences were observed in the age of cART initiation and type of childbirth, with increased frequency of cesarians in EARLY group. Regarding the type of prophylaxis received, EARLY group was enriched in prophylaxis of triple-drug combination Zidovudine/Lamivudine/Nevirapine (AZT, 3TC, NVP), a recommended guideline for high chances of perinatal HIV infection. No differences were found in breastfeeding, cART regimen at sampling, clinical category or other clinical parameters such as CD4+ and CD8+ cell counts, nadir CD4+ cell count and CD4+/CD8+ ratio.

Biochemical and inflammatory biomarkers including plasma glucose, GPT (ALT), GOT (AST), bilirubin, total cholesterol, triglycerides, HDL, LDL, and creatinine at sampling showed no differences between both groups (**Table S1**). Despite no differences between groups, LDL/HDL ratio correlated with age at cART initiation (ρ=0.74, p=0.001), and the levels of plasma triglycerides was positive associated with both the age at cART initiation (ρ=0.53, p=0.019) and age at sampling (ρ =0.51, p=0.03).

Differences in proteomic profile comparing early versus late cART initiation

A total of 188 proteins in plasma were identified and quantified using a shotgun proteomic approach. Out of them, seven were found significant different when comparing both groups and the PCA based on their expression level showed a clear cluster differentiation between the studied groups (**Figure 1, A-B**). The proportion of the variability regarding the first principal component (PC1) and the second principal component (PC2) was 17.33% and 44.63%, respectively.

Specifically, transthyretin (TTHY), a thyroid hormone-binding protein related to transport of thyroxine, and zinc-α-2-glycoprotein (ZA2G), a protein that stimulates lipid degradation in adipocytes, were down-regulated in EARLY group (FC=-1.269, p=0.012 and FC=-1.459, p=0.023, respectively). Contrarily, Complement component C9 (CO9) (FC=1.375, p=0.002), a constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune, Lysozyme (F8VV32) that is responsible of the hydrolysis of beta-linkages in peptidoglycans and chitodextrines (FC=1.283, p=0.029), Blood group Rh(CE) polypeptide (HOYCJ8) (FC=1.400, p=0.024) which is likely to have a transport or channel function in the erythrocyte membrane, Dipeptidyl peptidase 4 (DPP4) (FC=1.150, p=0.027) which is a Cell surface glycoprotein receptor involved in the costimulatory signal essential for T-cell receptor (TCR)-mediated T-cell activation, and Scm-like with four MBT domains protein 2 (SMBT2) (FC=1.305, p=0.007) that is a transcriptional repressor of HOXB13 gene are the five up-regulated proteins in EARLY group compared to LATE group.

Finally, these differentially expressed proteins were selected as input in the STRING database to predict protein-protein interactions and to establish which biological processes and pathways could be affected (**Figure 2A**). Of interest, five of these proteins (nodes) revealed a network of predicted functional associations (edges). In the biological process category, different processes related to immune response were one of the most-enriched terms where more than three of these seven selected proteins were associated (**Figure 2B**).

Associations between plasma lipids and protein levels

The lipidomic analysis identified and quantified 115 plasma lipids but we found no significant differences among groups. However, strong and significant correlations were

found in all participants between lipid species and plasma proteins. Of interest, the Complement component C9 was directly associated with the levels of plasma Cholesterol esters (ChoE16:1, ChoE18:1), Phosphatidylcholines (PC31:0, PC33:1) and Sphingomyelins (SM32:0, SM32:2, SM41:1) (**Figure 3, A-C**). Interestingly, these correlations were also found in the group of patients of EARLY cART after segregating by groups (data not shown). The same analysis revealed positive and strong associations in the case of Zinc-α-2-glycoprotein with PC33:2 and PC35:4, and triglycerides (TG47:1, TG56:5 and TG58:8) levels (**Figure S2**). In that case, only association with some lipids remain significant in EARLY cART group after segregating by groups (data not shown).

To investigate the discriminatory power in distinguishing between EARLY and LATE subjects, the AUC, as revealed by ROC, was calculated for each single test and the ratio among different compounds identified in proteomics and lipidomics. The CO9 reached highest values for discriminatory power, closely followed by TTHY, ZA2G, Lysozyme and H0YCJ8 proteins. In fact, the CO9 protein in combination with other proteins and lipids showed the highest discrepancy between groups, and indeed TTHY/CO9 and ZA2G/CO9 ratios showed higher discriminatory power than CO9, TTHY or ZA2G alone (**Figure 4**).

CD4 and CD8-T cell counts, age and time under cART are associated with plasma metabolite levels

Regarding plasma metabolites, no differences were observed comparing groups in none of the 81 quantified plasma metabolites but interestingly, multiple correlations were found between metabolites and clinical parameters (**Figure 5**). Serine and methionine amino acid levels and the Krebs cycle intermediate, methylmalonic acid, were inversely

associated with CD4+ and CD8+T cell counts. On the other hand, 4-hydroxybenzoic acid and the monosaccharides D-arabitol, D-ribose and D-sucrose were positive related to CD4+ and CD8+ T cell counts. Besides, all those metabolites strongly correlated with age and time under cART at sampling. The correlations with the amino acids serine and methionine and the methylmalonic acid were positive, in contrast with the inversed associations found between 4-hydroxybenzoic acid and monosaccharides, and age and time under cART. Again, the AUC was used to evaluate the discriminatory power in distinguishing between EARLY and LATE subjects. But, none of the metabolites alone or combined with other proteins and lipids resulted to be discriminatory among groups.

Discussion

In the present study based on a cross-sectional retrospective design of HIV-infected children followed up during a median of 11 years since birth, we have found a specific plasma profile that characterizes perinatally HIV-infected children who initiated cART during the first 12 weeks of life compared with HIV-1 children who started the cART latter, between 12 and 50 weeks after delivery. Marked differences between the age at treatment initiation corroborate that the earlier the start of treatment in children, the greater the benefits for their health when they become adults.[4,5]

It is well known that one of the consequences of a late cART initiation is the increased HIV-1 viral reservoir.[10,13] In that sense, HIV-1 replication, even at very low level, results in changes in cell cycle, morphology, cellular signalling and host protein synthesis dysregulation and degradation, so it was expected to find alterations in the protein profile of the study patients as we have demonstrated in this work. The recognition of factors and biomarkers associated with limited viral reservoir size are critical to identify study participants for proof-of-concept studies aimed at curing HIV infection.

Lysozyme levels were significantly higher in the group who started an early cART. Interestingly, anti-HIV activity has been attributed to this enzyme and the activity mapping revealed two peptides (HL18 and HL9) able to block HIV-1 viral entrance and replication, in addition to modulate gene expression of HIV-infected cells, affecting multiple pathways including those involved in survival, stress and NFkappaB,[29,30] and also confirmed by the enriched biological processes. This antiviral activity may explain the low viral reservoir associated with this population of perinatally-acquired HIV-1

infected children who started early cART. Interestingly, lysozyme recombinant protein has been also considered useful for the design of future anti-HIV-1 therapeutic agents.[31]

Additionally, HIV-infected children who initiated cART during the first 12 weeks of age had significant lower levels of transthyretin (TTHY). This is not in accordance with previous observations in which lower levels of TTHY were associated with higher HIV morality and advance immune suppression.[32,33] Differences in ethnicity and age, being the majority of previous studies carried out in adults, may explain the lack of accordance with our results. However, despite no statistical differences in the retinol-binding protein (RBP), also identified and quantified in our proteomic approach, we determined the RBP/TTHY ratio, proposed as an indirect method to determine vitamin A status and its levels had been associated with advance HIV and negative acute phase response.[34] However, no significant differences in RBP/TTHY ratio were found in comparing groups (p=0.151).

Zinc α-2-glycoprotein (ZA2G) levels were lower in the EARLY group. This protein is implicated in dyslipidaemia in HIV-1 infected patients and it is also associated with plasma lipids.[35] In fact, our results highlight a strong and significant association between some plasma protein levels and plasma lipid concentrations. Concretely, strong positive correlations were found in all participants between ZA2G and plasma lipid components and a borderline statistical significance towards a lower ratio LDL/HDL. In HIV-perinatally infected children and adolescents, dyslipidaemia has been associated with the time under suppressive cART and the type of antiretroviral regimen especially those combinations with protease inhibitors.[36–38] In our work, both groups of patients showed no differences in the duration under cART and type of cART regimen at the moment of the study. Thus, our results suggest that a dyslipidaemia profile related to a

delay in the cART setting, independent on cART regimen, may develop future cardiovascular comorbidities.

The ratio of TTHY and ZA2G with complement component C9 (TTHY/CO9 and ZA2G/CO9) showed the most discriminatory capacity to distinguish both groups of patients. Complement system is dysregulated due to HIV infection and it contributes to a proinflammatory state even in well-controlled HIV infection.[39] Besides, complement proteins, including CO9, have been highlighted in the pathogenesis of HIV-1 related neurodegenerative disorders and cognitive impairment in adults.[40,41] Contrary to those observations, in our study CO9 levels were increased in EARLY cART group. Up to now, there are limited data available on cognitive differences associated with HIV and cART exposure children.[42] Further follow-ups and carried out future studies once those perinatally HIV-infected patients reach adulthood will determined the possible implication of CO9 in the development of cognitive disorders due to an early cART introduction. In fact, the expected interaction among TTHY-Lysozyme resulted in the enrichment of amyloid fibre formation, structures based on misfolded proteins that are linked to multiple neurodegenerative disorders. In this regard, it is known that membranes containing phosphatidylserine (PS) due to a variety of proteins, which include lysozyme or transthyretin, induce a rapid formation of amyloid fibers.[43]

Regarding the metabolomic and lipidomic profiles, the study of latently HIV-infected T-cells and macrophages has identified several unique metabolic features of these cells.[22,44] Thus, the targeting of specific metabolic pathways of viral reservoirs has been considered as a promising therapeutic approach to eradicate viral reservoirs in HIV infected individuals. In our study, the lack of differences between groups in the metabolomic and lipidomic profiles could be explained by the demanding matching by most clinical parameters between groups, including the type of childhood nutrition [45].

This fact, together with the absence of data regarding HIV-1 latency in our groups of children, does not allow us to identify a specific metabolic signature associated with viral reservoir. Indeed, concentrations of metabolites are really dynamics and metabolomics can only take a snapshot in time under a set of defined conditions. Interestingly, despite no differences in CD4-, CD8-T cells, age and time under cART, some metabolites involved in the glucidic metabolism strongly correlated with those clinical parameters previously observed in HIV-1 infected adults whose immune system and metabolic imbalance of chronic HIV-1 infection is aggravated by the cART toxicities and, in fact, the time of exposure as previously described.[23,46,47] Impaired glucidic metabolism could develop insulin resistance and diabetes, significant risk factors of cardiovascular diseases, but none participant in our study had been diagnosed of any metabolic syndrome before and after the sampling analysis. In the case of amino acid levels, intestinal epithelial barrier disruption due to chronic HIV infection may alter amino acids absorption [48,49] and several studies had focused on the impact of cART on metabolism in perinatally HIV-infected children.[50,51]

In our study, data concerning HIV-infected mothers, including viral load at the moment of childbirth, was limited: many of them were born in other countries and the hospitals for the new-borns follow-up does not always coincide with the hospital at birth.[52] However, EARLY group was enriched in cesarians and triple-drug regimen (AZT/3TC/NVP) as prophylaxis, recommended guideline for high chances of perinatal HIV infection, suggesting that in those cases, mothers had probably high levels of viral load.

The limitations of this study such as a cross-sectional design, the relatively scarce number of participants (10 children per group) and the lack of a group of non-HIV children as a control group, made difficult to draw conclusions and that does not allow

inferring in a future clinical outcome. This limitation is counterbalanced by the exhaustive follow-up of the HIV-infected patients during a median of 11 years since birth. In this period none of them have develop any inflammatory disease or non-AIDS-event like cardiovascular or metabolic disorders. Further longitudinal observation would be needed to determine clinical consequences that could be explained by the late start of cART. According to our inclusion criteria, the two groups of patients included in our study had been treated during the first year of life. Perhaps, a design with groups of patients separated with higher timing of ART initiation would point out to more differences. Also, the inclusion of pre-cART time-points should be considered to obtain a more discernible signature from, either the virus and/or the cART influence. Finally, an appropriately powered study would be welcomed for the validations of the results.

In conclusion, this is the first extensive analysis of the proteomic, lipidomic and metabolomic profile in plasma in perinatally HIV-infected children during their childhood that has identified a distinct protein signature associated with the time of cART initiation. The profile of late cART introduction shows a proinflammatory state, also associated with dyslipidemia and atherogenic components that may potentially trigger premature non-AIDS events including atherosclerotic diseases and metabolic disorders. Developing therapeutic strategies targeting protein and metabolic abnormalities may be beneficial for preventing future complications in HIV-infected children.

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Authors' contributions

FV and MªAM-F conceived and coordinated the study development. LT-D, AR and EV-A designed, performed experiments, analysed and interpreted the data, designed the figures and wrote de manuscript. AR and PH contributed in sample preparation and in mass spectrometry analysis. JP, SG, MLN-G and CV collaborated with patient's characterization and sample collection. All authors critically reviewed, edited and approved the final version of the manuscript.

Transparency declarations

None to declare.

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Figure legends.

Figure 1. A) Principal Component Analysis (PCA) score plot representation of protein profile comparing EARLY and LATE cART initiation. The plot shows the two principal components that include PC1=44.63% and PC2=17.73% percentage of total variables. **B**) Differential plasma proteins using log2 (normalized) among groups. Abbreviations: CO9, Complement component C9; DPP4, Dipeptidyl peptidase 4; F8VV32, Lysozyme; HOYCJ8, Blood group Rh(CE) polypeptide; SMBT2, Scm-like with four MBT domains protein 2; TTHY, transthyretin and ZA2G, zinc-alpha-2-glycoprotein.

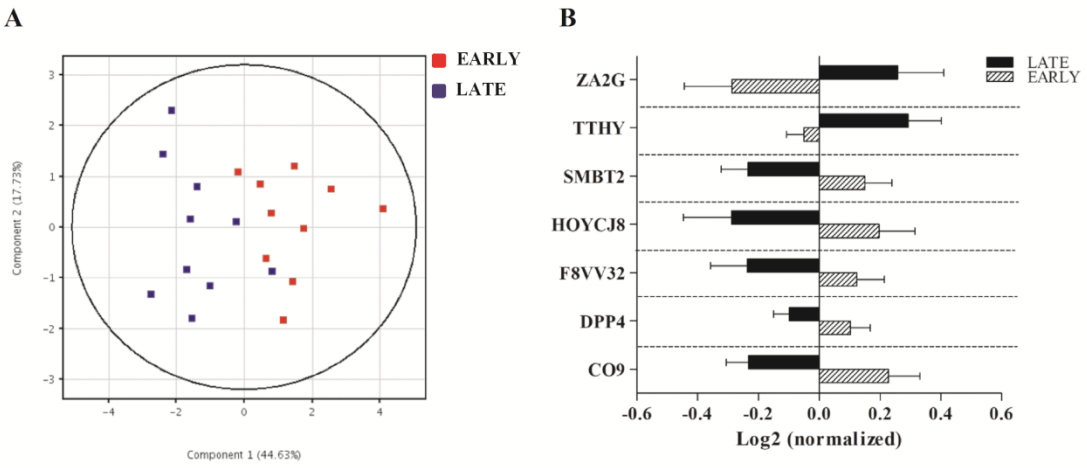


Figure 2. Protein-Protein interaction analysis using the STRING database. The seven differentially expressed proteins have been selected as input, expanded by an additional 10 proteins in the STRING interface and the confidence cut-off for showing interactions links has been set to medium confidence (0.400). The STRING database identified different functional enrichments associated with our network using the false discovery rate (FDR) (p-values were corrected for multiple testing within each category using the Benjamini–Hochberg procedure) (A). The biological process and KEGG pathways with more than two of the seven selected proteins annotated with a particular term were showed (B).

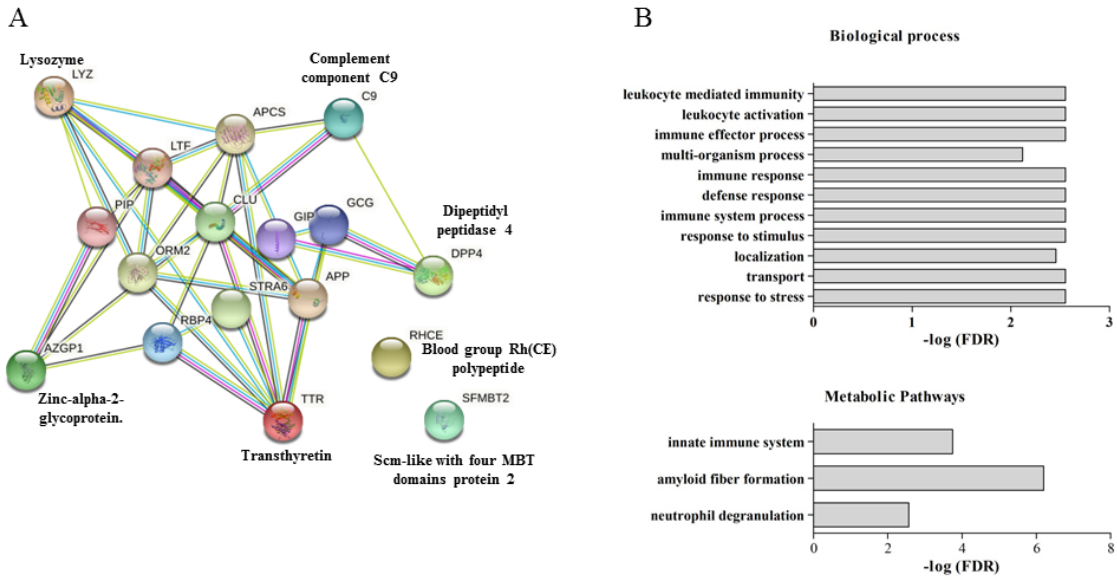


Figure 3. Associations between plasma lipids and the Complement component C9. Correlations with the levels of Cholesterol esters (ChoE) (A), Phosphatidylcholines (PC) (B) and Sphingomyelins (SM) (C) including 19 participants with available data. The Spearman ρ correlation coefficient test was used.

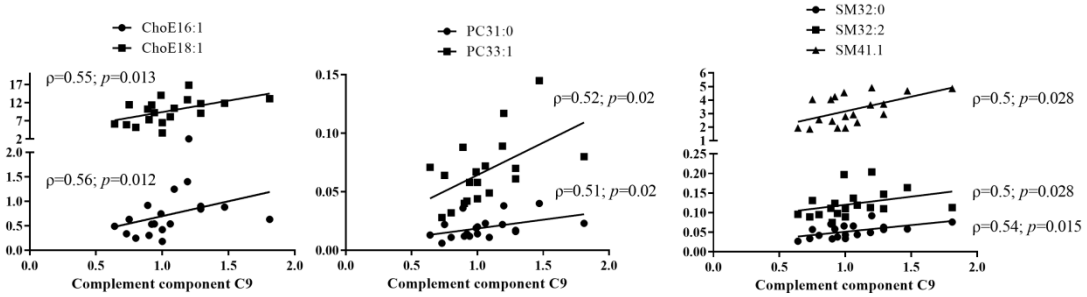


Figure 4. Discriminatory power in distinguishing EARY and LATE subjects as measured with the Areas Under the Curve (AUC) as revealed by Receiver operating characteristic curves (ROC) calculated for both single proteins and the ratio between protein and lipids. The specificity and sensitivity of each parameter is indicated in continued and dashed lines, respectively.

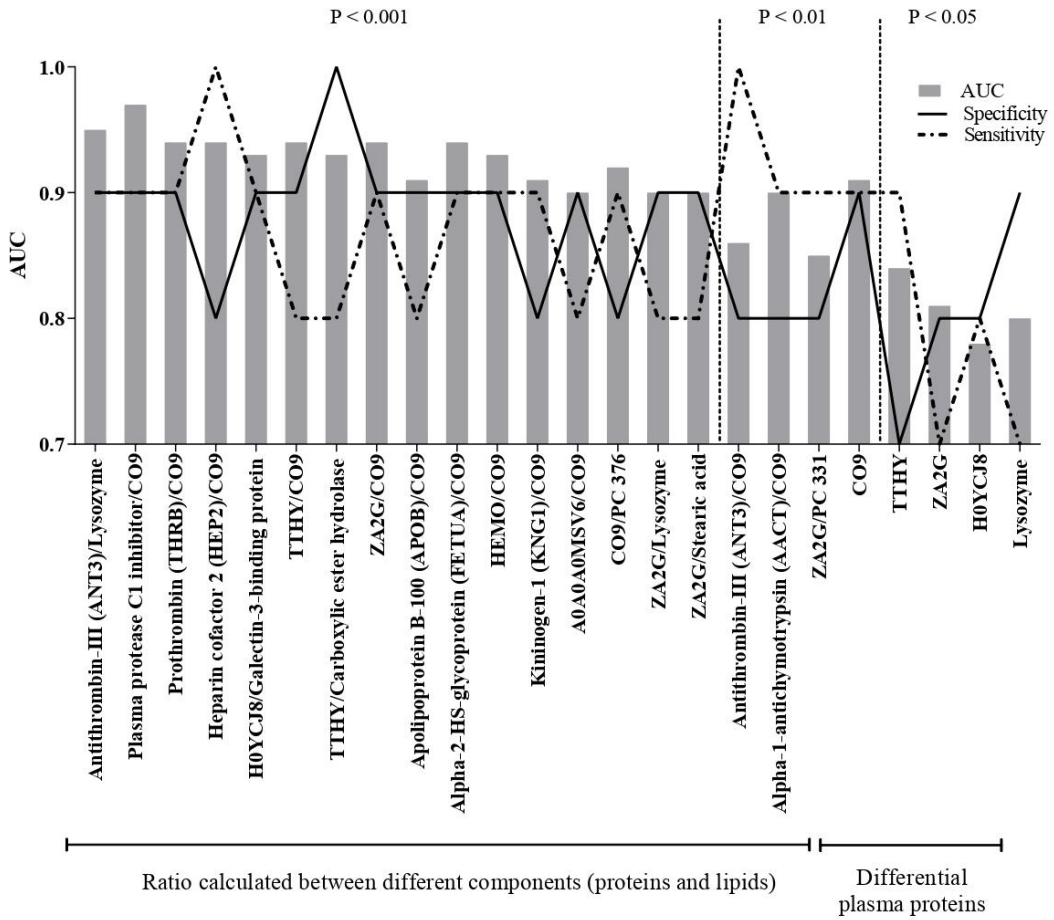
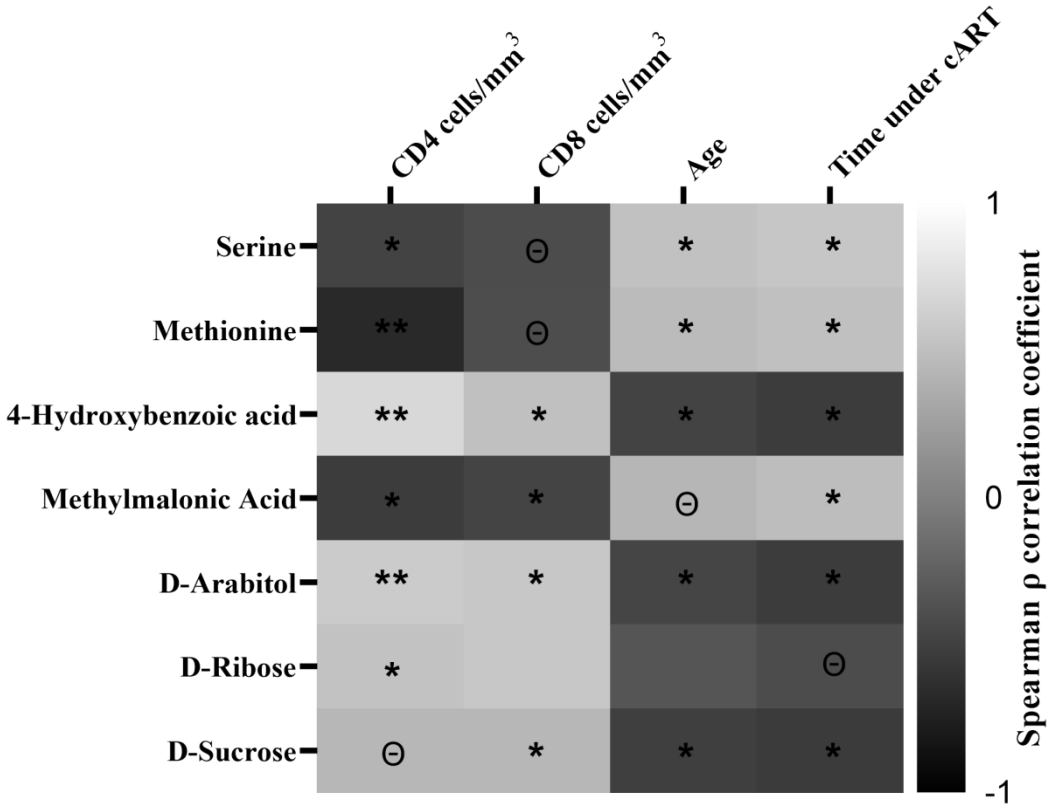


Figure 5. Asssociations between clinical parameters and plasma metabolites. . Clinical parameters are taken at sampling. Spearman ρ correlation coefficient test. $^{**}p < 0.001$, $^{*}p < 0.05$, $^{\Theta}p < 0.1$. Only correlations with $p<0.1$ are shown.



598 **Table 1.** Study cohort characteristics.

	Treatment Initiation		
	EARLY (0-12 weeks) n=10	**LATE (12-50 weeks) n=10**	***p***
Subject characteristics			
Age at cART initiation (wk)	5 [0-9]	30 [24-34]	**<0.000**
Sex (male) n. (%)	1 (10)	5 (50)	0.051
Nadir CD4+ cells/mm^3	703 [397-843]	545 [342-751]	0.384
Type of childbirth (cesarian) n. (%)[a]	7 (70)	2 (22.2)	**0.037**
Received prophylaxis (yes) n. (%)	7 (70)	6 (60)	0.639
Type of prophylaxis n. (%)[±]			**0.041**
AZT	1 (14.3)	5 (83.3)	
AZT 3TC NVP	6 (85.7)	1 (16.7)	
Breastfeeding (yes) n. (%)[a]	2 (20)	2 (22.2)	0.906
Parameters at sampling			
Age (years)	6.4 [4.8-7.9]	8.5 [6.1-12.8]	0.096
Time since cART initiation (wk)	76 [58-92]	94 [67-146]	0.162
Time under virologic control (wk)	65 [47-86]	87 [60-139]	0.131
Time since HIV diagnosis (years)	6.0 [4.7-7.7]	7.5 [5.0-11.5]	0.218
n CD4+ cells/mm^3	1203 [977-1783]	1235 [645-1662]	0.705
n CD8+ cells/mm^3	707 [549-913]	711 [508-1016]	0.940
Ratio CD4+/CD8+	1.75 [1.51-2.05]	1.68 [1.06-2.04]	0.821
cART regimen n. (%)			0.639
NRTI + IP	3 (30)	4 (40)	
NRTI + NNRTI	7 (60)	6 (60)	
Clinical category			0.303
Mild %)	9 (90)	6 (60)	
Moderate or severe (%)	1 (20)	4 (40)	

[±]Percentage calculated with respect to the children that received prophylaxis. [a] One child from LATE treated group with no information about type of childbirth and breastfeeding. Clinical category A was considered as mild symptomatic state. Clinical categories B and C were considered as moderate and severe symptomatic states. Values are show as median [IQR] for continuous variables or num (%) for categorical variables. Mann-Whitney test was used for comparisons between continuous variables. Chi-square or Fisher tests were used for comparisons between categorical variables. Abbreviations: cART, combined antiretroviral therapy; wk, weeks; AZT, Zidovudine; 3TC, Lamivudine; NVP, Nevirapine; NRTI, nucleoside analogue reverse-transcriptase inhibitors; IP, protease inhibitors and NNRTI, non-nucleoside analogue reverse-transcriptase.