Predicting adverse events during therapy for HIV and hepatitis C

The role of ITPase activity and *ITPA* genotype

Nicole Chantal Peltenburg

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Predicting adverse events during therapy for HIV and hepatitis C

The role of ITPase activity and *ITPA* genotype

Het voorspellen van bijwerkingen tijdens therapie voor HIV en hepatitis C

De rol van ITPase activiteit en *ITPA* genotype

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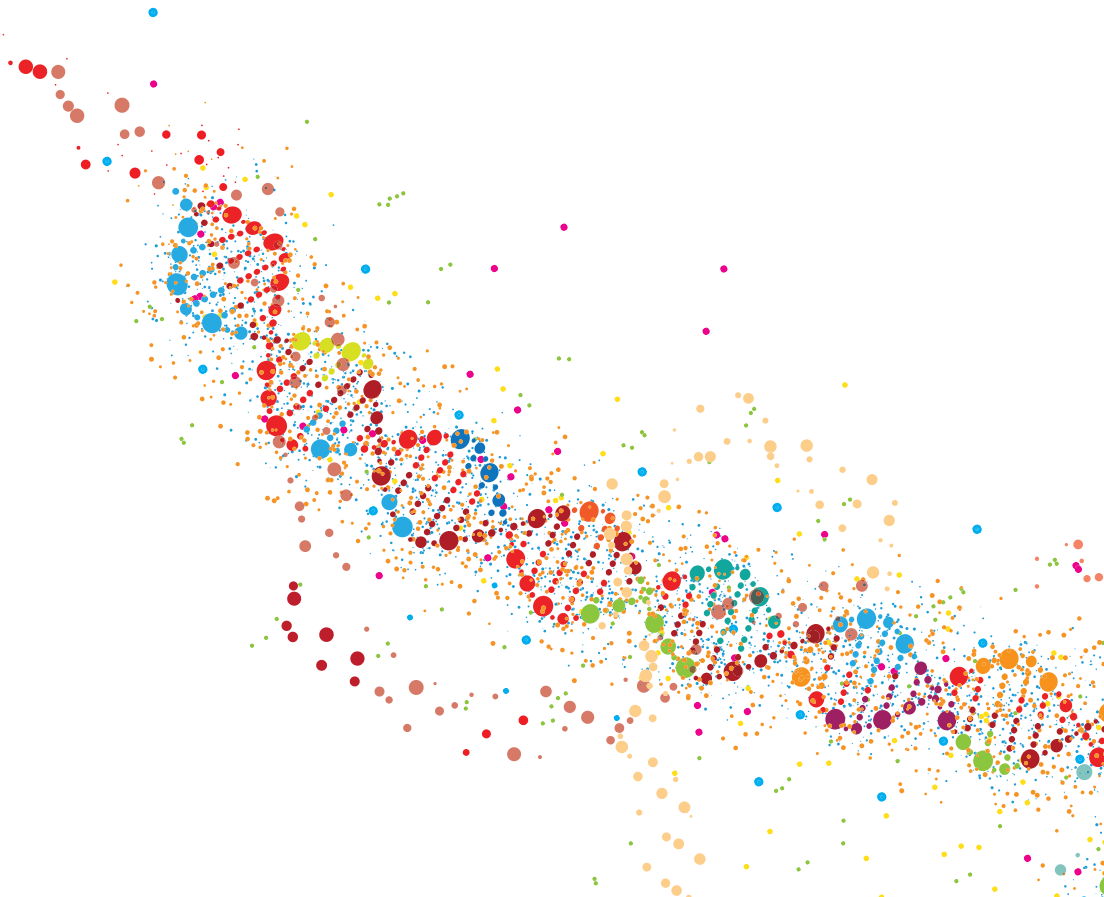
Chapter 1.

General introduction and outline of the thesis



HUMAN IMMUNODEFICIENCY VIRUS

In 1983 the Human immunodeficiency virus (HIV) was discovered after the clinical observation in 1981 of a cluster of patients who were suffering from *Pneumocystis jirovecii* (previously carinii) pneumonia.[1] This rare opportunistic infection was only reported in patients with a severely impaired immune system, but this cluster of patients did not have a medical history of immune deficiency. In 2017, there were an estimated 36.9 million (31.1-43.9 million) people living with HIV worldwide and in July 2018 21.7 million (19.1-22.6 million) of those people had access to antiretroviral therapy.[2]

The HIV is a single-stranded ribonucleic acid (RNA) virus, that specifically infects $CD4^+$ T-helper lymphocytes, macrophages and dendritic cells by binding of glycoprotein 120, a protein on its trimeric envelope complex, to the CD4-receptor and one of the co-receptors CCR5 or CXCR4 on the target cell.[3-5] After a series of steps, HIV glycoprotein 41 is inserted into the host cellular membrane and then undergoes a significant conformational change, forming a hairpin and bringing the membranes of the HIV and the host cell together, thereby allowing them to fuse.[6] After the fusion process, the HIV capsid enters the cytoplasm of the target cell, where it releases its content: the enzymes reverse transcriptase, integrase and protease, some minor proteins, the major core protein and two single-stranded RNA strands.[7] Inside the cytoplasm, HIV uses its enzyme reverse transcriptase and the nucleotides of the host to transform its single-stranded RNA into a double-stranded deoxyribonucleic acid (DNA) molecule.[8] This DNA is transported to the nucleus of the host cell and via the enzyme integrase incorporated into the DNA of the host.[9] The nucleotide metabolism of the host further transcribes this incorporated DNA into new RNA strands, that, after translation of new essential HIV proteins, are assembled together with these proteins into a new HIV virion that buds from the host membrane and is released from the host cell.[7] Finally, the HIV enzyme protease completes the last step of the HIV cycle, maturing the virus to a new infectious particle. The HIV-cycle is displayed in Figure 1.

If left untreated, HIV infection eventually leads to a depletion of $CD4^+$ T-lymphocytes. The mechanism behind the slow decrease of $CD4^+$ T-lymphocytes is not completely understood, but probably consists of a combination of factors. HIV is causing a direct cytotoxicity, leading to massive $CD4^+$ T-cell destruction, occurring in the early course of the infection. It is thought that this massive destruction is followed by a regenerative response of $CD4^+$ memory T-cells, preserving $CD4^+$ T-cell numbers and immune function. However, this homeostatic balance eventually fails, due to factors such as infection and death of progenitor cells,[10] destruction of the secondary lymphoid tissues that support the homeostasis[11] and chronic inflammation. The chronic inflammation is also sustained by a combination of factors, like gastrointestinal mucosa loss (leading to microbial translocation),[12] pyroptosis (a

highly inflammatory form of programmed cell death like apoptosis, leading to the release of the dying cells contents among which inflammatory cytokines)[13] and polyclonal activation of B-cells[14] and pro-inflammatory cytokines.[15] Failing of the homeostasic balance between $CD4^+$ T-cell destruction and regeneration, leads to a critical effector T-cell population loss,[16] resulting in the Acquired Immune Deficiency Syndrome (AIDS). AIDS is the last stage of HIV-infection, defined by a $CD4^+$ T-cell count below 200 cells/mm^3 or the presence of an AIDS-defining illness, which are opportunistic infections or certain types of cancer, and ultimately death. However, this lethal chain of events can be reversed by combination antiretroviral therapy; cART. Antiretroviral therapy has become increasingly effective over the last decades in inhibiting the replication of HIV and enabling the restoration of the immune system. The drugs used in antiretroviral therapy for HIV can be categorized as CCR5 antagonists, fusion and entry inhibitors, non-nucleoside reverse transcriptase inhibitors (NNRTI), reverse transcriptase inhibitors that are nucleoside/nucleotide analogues (NRTI), integrase inhibitors (INSTI) and protease inhibitors (PI), based on their target in the HIV replication cycle (Figure 1). A cART regimen consists of a combination of drugs from different classes. According to all commonly used guidelines[17-21] the preferred initial treatment for all cART naïve patients consists of two NRTIs combined with either an NNRTI, a PI with a pharmacoenhancer (also called a booster) or an integrase inhibitor. Very recent studies, however, have shown equal effectiveness with combinations of 2 drugs instead of 3, and thus the guidelines might be adapted on short notice.[22-24]

After the introduction of cART, the life-expectancy of people living with HIV is now approaching the life-expectancy of the non-HIV infected general population in the Western world.[25] Additionally, cART significantly reduces new HIV transmissions to HIV-negative sexual partners either by treatment of the HIV-positive partner (Treatment as prevention; TASP)[26,27] or by treating the HIV-negative partner prior to sexual intercourse (Pre-exposure prophylaxis; PREP).[28] Although these therapeutic strategies are highly effective, cART is not curative and cessation leads to a rebound of HIV viremia. Treatment of HIV requires a lifelong commitment to medication with the risk of adverse effects, sometimes severe, which have been reported for all drugs used in cART regimens. Besides HLA-B*57:01, a marker to predict hypersensitivity for abacavir, no other biomarkers or genetic susceptibility traits are known to predict the occurrence of adverse events during cART use.

Thus HIV treatment needs to be further ameliorated, in order to diminish adverse reactions during cART use, and new biomarkers to predict adverse reactions would be very helpful tools. Both the replication of HIV and the mechanism of action of the NRTIs, the backbone of the cART regimens currently recommended in the HIV treatment guidelines, depend for an important part on the human nucleotide metabolism. Further, also in other viral infections, like hepatitis C (HCV), hepatitis B (HBV) and herpesviridae, nucleotide analogues

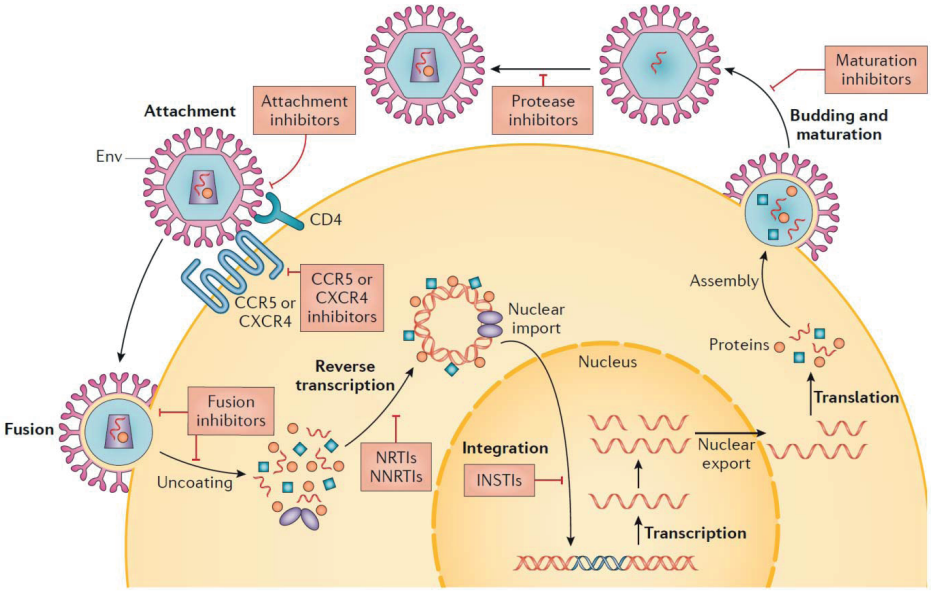


Figure 1. HIV replication cycle and drug targets for combination antiretroviral therapy. *Adapted from: Deeks, S.G.; Overbaugh, J.; Phillips, A.; Buchbinder, S.; HIV Infection. Nature Reviews Disease Primers, October 2015; Vol 1; p1-22. Licensed by Springer Nature RightsLink.*

are used in the treatment. Knowledge regarding the nucleotide metabolism is therefore important to improve inhibition of HIV replication and decrease adverse events during therapy with nucleotide analogue drugs in HIV and various other infectious diseases.

HUMAN NUCLEOTIDE METABOLISM

Canonical nucleotides

Nucleotides are the building blocks of DNA and RNA, and the basis for the function of all cells in all living species (including humans). In humans, DNA is located in the nucleus (chromosomal DNA) and in the mitochondrion (circular mtDNA). Nucleotides contain a nucleobase, a deoxyribose (together a nucleoside) and a phosphate group (Figure 2a). In RNA the nucleoside contains a ribose instead of a deoxyribose.

DNA is composed of 2 strings of nucleotides, bound together via the nucleobases by hydrogen bonds and forming the genetic code (Figure 2b). The nucleobases incorporated in nucleic acids are adenine, guanine, cytosine and thymine, and are considered the canonical nucleobases. These nucleobases can be divided in 2 groups according to their chemical structure. Cytosine and thymidine (and uracil in RNA) are pyrimidines and have a core in

the shape of a pyrimidine ring (C4H4N2). Adenine and guanine are purines and have a core consisting of a heterocyclic aromatic ring (with the same structure as the pyrimidine ring) fused to a imidazole ring (C3H4N2) (Figure 3).

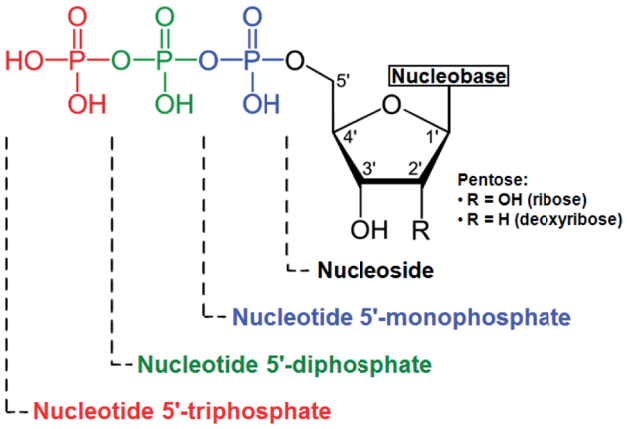


Figure 2a. Chemical structure of nucleoside and nucleotide 5'-mono-, di- and triphosphate. *Adapted from: Yikrazuul, own work, general overview of nucleotides and nucleosides, may 26th 2010, URL https://commons.wikimedia.org/wiki/File:Nucleotide_nucleoside_general.svg, accessed June 28th 2018.*

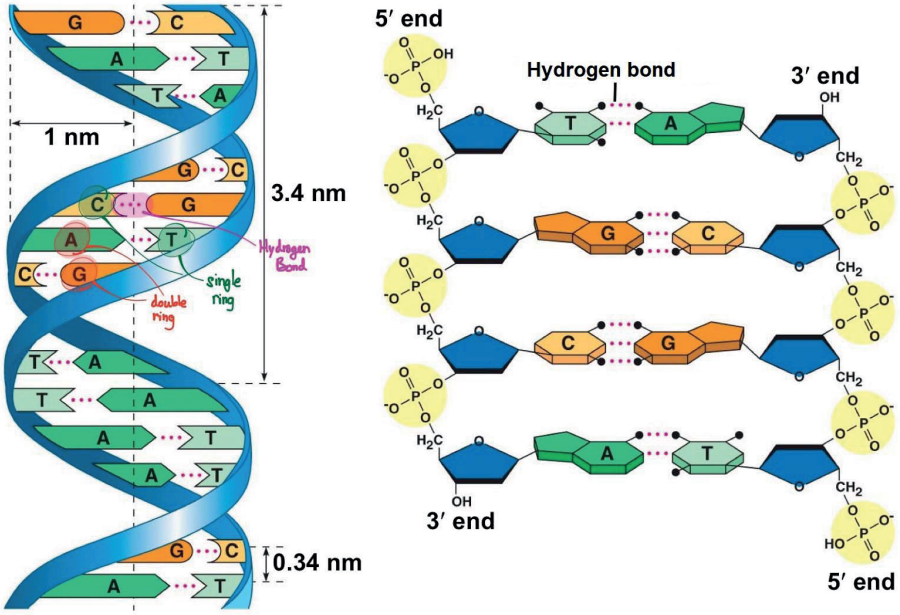


Figure 2b. Schematic DNA structure (left) and nucleobase bonds (right). *Adapted from: Pearson Education, Inc., publishing as Pearson Benjamin Cummings, URL http://logyofbio.blogspot.com/2016/02/structure-of-dna-and-nucleotides.html, accessed June 28th 2018.*

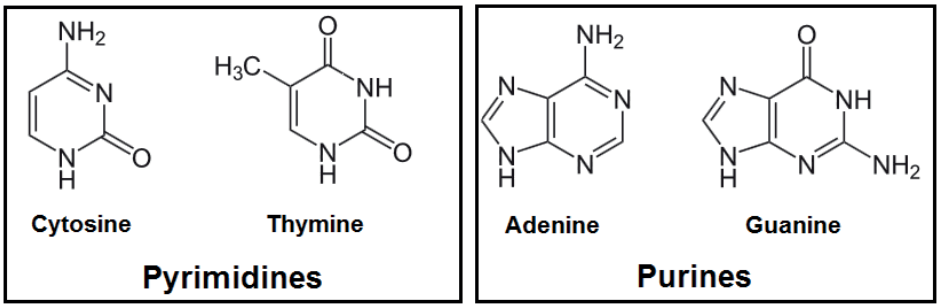


Figure 3. Chemical structure of the canonical pyrimidines and purines.

Role of canonical nucleotides in human metabolism

Besides being building blocks for DNA, the nucleotides have additional important functions in the human cell metabolism. The nucleotide-5'-triphosphates function as energy carriers because energy is released when the phosphate groups are hydrolysed from the nucleoside base by kinases. From the nucleotide-5'-triphosphates, adenosine 5'-triphosphate (ATP) is the most preferred nucleotide for most cellular processes. The preference for ATP has been observed in a large study investigating over 200 kinases and found that most had affinity for ATP and only a small number of the kinases exhibited affinity for guanosine 5'-triphosphate (GTP).[29] Further, cyclic nucleotides act as secondary messengers, transducing signals from outside the cells to intracellular. For example: the primary messenger epinephrine stimulates the liver cell via triggering the secondary messenger cyclic adenosine 3'5'-monophosphate (cAMP) to convert glycogen to glucose (glycogenolysis).[30] cAMP is generated from ATP by adenylyl cyclase and has three major targets in most cells: protein kinase A (PKA), exchange proteins activated by cAMP (Epacs) and cyclic-nucleotide-gated ion channels.[31] PKA phosphorylates numerous metabolic enzymes, among which enzymes regulating glycogen, sugar and lipid metabolism, depending on the cell type. The Epac proteins are involved in multiple cellular functions such as (among others) cell adhesion, cell differentiation, apoptosis and secretion.[32-34] Cyclic guanine 3'5'-monophosphate (cGMP), produced from GTP by two families of guanylyl cyclases: transmembrane particulate guanylyl cyclase (pGC) and soluble guanylyl cylclase (sGC), activate protein kinase G (PKG). PKG phosphorylates several enzymes responsible for multiple cellular processes, like vascular tone and remodeling, neuronal adaptation, intestinal water secretion and bone growth.[35] cGMP modulates cAMP concentration[36] and, like cAMP, also regulates cyclic nucleotide-gated ion channels.[37] Nucleotides are also important constituents of the coenzymes nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and Flavin adenine dinucleotide (FAD), which play a role in oxidation-reduction reactions. And finally, nucleosides are involved in the synthesis of polysaccharides, for instance UDP-glucose which is an intermediate in the glycogen synthesis in mammals.[38]

Purine nucleotide synthesis

In the human metabolism two pathways of purine nucleotide synthesis exist: the 'de novo' synthesis pathway, in which the purine base is synthesized step by step on the ribose-5'-phosphate, and the salvage pathway, in which a ribose-5'-phophate is added to the preformed purine base or phosphates are added to a preformed, or rather recycled, purine nucleobase. In the de novo purine synthesis pathway (Figure 4), inosine 5'-monophosphate (IMP) is formed from the active form of ribose: 5-phosphoribosyl-1-pyrophosphate (PRPP) through 10 enzymatic steps. IMP is further converted into either adenosine 5'-monophosphate (AMP) or guanosine 5'-monophosphate (GMP) in two final steps (Figure 5). To form AMP, first the carbonyl oxygen atom at C6 is substituted for an amino group on which aspartate is added by the enzyme adenylosuccinate synthetase. Finally, fumarate is released from the amino group by adenylosuccinase. GMP is formed by oxidation of IMP at C2 by IMP dehydrogenase using NAD^+ and H2O, followed by transfer of the amido-N of glutamine to the C2 position by GMP synthetase. Note that for the synthesis of AMP, GTP is used and for the synthesis of GMP, ATP is used.

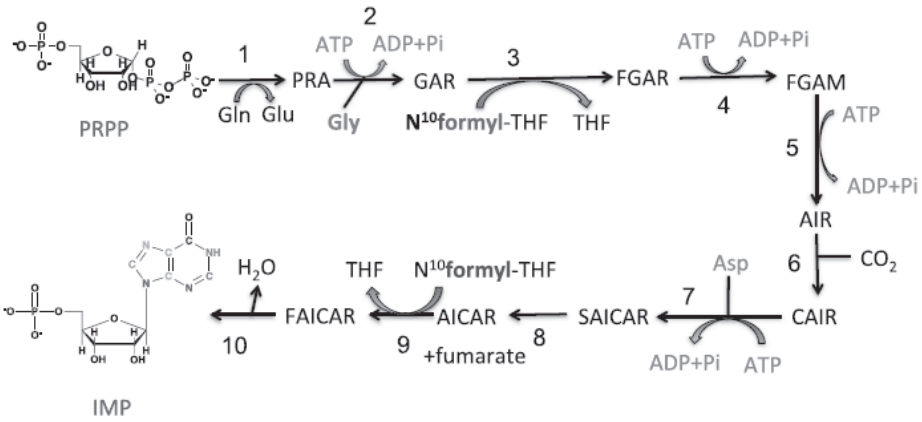


Figure 4. Purine nucleotide de novo pathway. Inosine 5'-monophosphate (IMP) is formed from 5-phosphoribosyl-1-pyrophosphate (PRPP) in 10 enzymatic steps: (1) glutamine phosphoribosylpyrophosphate amidotransferase (GPAT activity - *PPAT* gene); (2) glycinamide ribonucleotide synthetase (GARS activity - *GART* gene); (3) glycinamide ribonucleotide formyltransferase (GART activity - *GART* gene); (4) phosphoribosylformylglycinamide synthase (PFAS activity - *PFAS* gene); (5) aminoimidazole ribonucleotide synthetase (AIRS activity - *GART* gene); (6) aminoimidazole ribotide carboxylase (AIRC activity - *PAICS* gene); (7) succinylaminoimidazolecarboxamide ribonucleotide synthetase (SAICAR activity - *PAICS* gene); (8) adenylosuccinate lyase (ADSL activity - *ADSL* gene); (9) 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT activity - *ATIC* gene); (10) IMP cyclohydrolase (IMPCH activity - *ATIC* gene). *From: Lane A.N. and Fan T.W.-M. Regulation of mammalian nucleotide metabolism and biosynthesis. Nucleic Acids Research, 2015, Vol 43(4): p.2466-2485.*

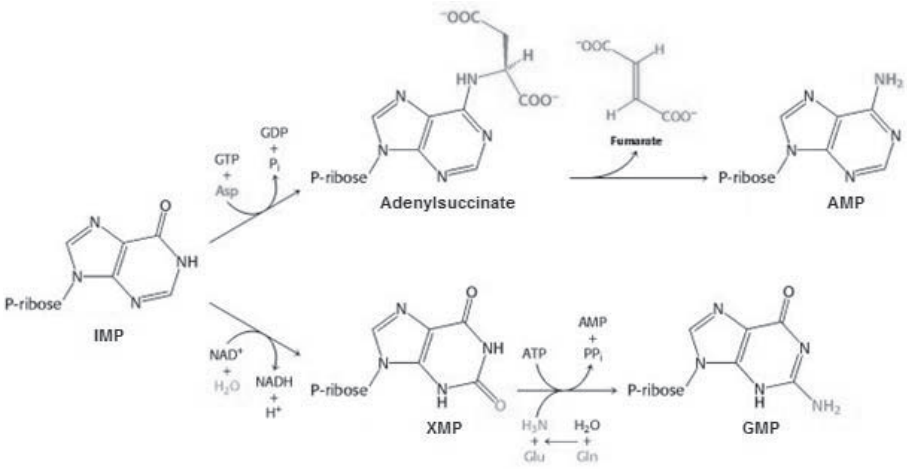


Figure 5. Interconversion of purines; synthesis of AMP and GMP from IMP. *Adapted from: Berg J.M., Tymoczko J.L. and Stryer L. Biochemistry. 5th edition 2002. New York: W.H. Freeman.*

In erythrocytes the 'de novo' pathway is absent, so the erythrocyte relies completely on the salvage pathway (Figure 6) for the requirement of the purine nucleotides. The salvage pathway provides a way to utilize purine bases derived from diet (exogenous) or from the normal turnover of nucleic acids (endogenous) and reconverts these purine bases to their corresponding nucleotides by phosphoribosylation. As in the de novo synthesis pathway, PRPP serves as the activated ribose-5-phosphate. The enzymes involved in the process of resynthesis of nucleotides from bases are adenine phosphoribosyltransferase (APRT) for adenine and hypoxanthine-guanine phophoribosyltransferase (HGPRT) for hypoxanthine and guanine. Thereafter, the purine nucleosides are degraded in several steps to the ultimate end product uric acid, which is excreted in the urine.

NRTIs in HIV

The mechanism of action of the NRTIs, used as backbone of the cART regimens currently recommended in HIV treatment guidelines, is based on the human nucleotide metabolism. Abacavir, tenofovir disoproxil fumarate (from now on referred to as tenofovir) and didanosine (which is currently no longer widely used) are NRTIs resembling the natural purine nucleosides (Figure 7); abacavir and didanosine being guanosine nucleoside analogues and tenofovir being an adenine nucleotide analogue.

These drugs are first metabolized inside the cells and converted to their active 5'-triphosphate forms. For instance, carbovir is the active 'triphosphate' form of abacavir. The active 5'-triphosphate forms compete with the natural purine nucleotide triphosphates for incorporation in the growing viral DNA, but due to the lack of a 3'-hydroxyl group, the next nucleotide cannot be added to the DNA strand and further DNA synthesis is terminated.

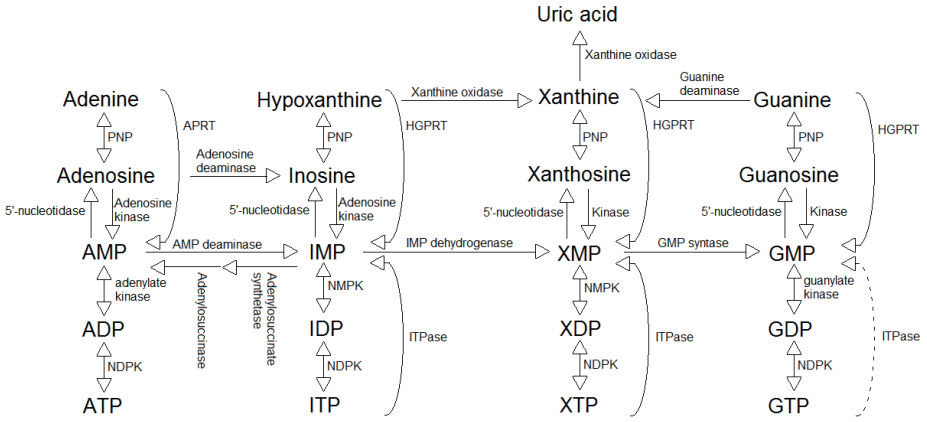


Figure 6. Purine salvage pathway. PNP, Purine nucleotide phosphorylase; APRT, Adenine phosphoribosyltransferase; HGPRT, Hypoxanthine-guanine phosphoribosyltransferase; AMP, Adenosine 5'-monophosphate; IMP, Inosine 5'-monophosphate; XMP, Xanthosine 5'-monophosphate; GMP, Guanosine 5'-monophosphate; NMPK, Nucleoside monophosphate kinase; ADP, Adenosine 5'-diphosphate; IDP, Inosine 5'-diphosphate; XDP, Xanthosine 5'-diphosphate; GDP, Guanosine 5'-diphosphate; NDPK, Nucleoside diphosphate kinase; ATP, Adenosine 5'-triphosphate; ITP, Inosine 5'-triphosphate; XTP, Xanthosine 5'-triphosphate; GTP, Guanosine 5'-triphospposphate.

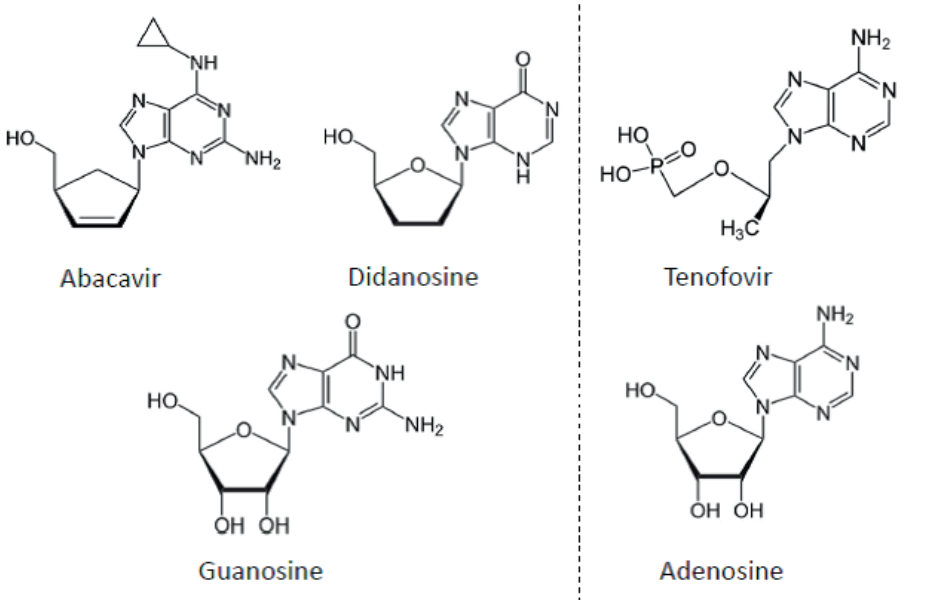


Figure 7. Chemical structures of the purine analogues non-reverse transcriptase inhibitors, abacavir, didanosine and tenofovir and their resemblance with the natural purine nucleosides guanosine and adenosine.

Non-canonical nucleotides

Besides the canonical purine nucleobases (adenine and guanine), other non-canonical nucleobases exist that are not building blocks of the genetic code. For instance, xanthine and hypoxanthine are important non-coding intermediates in purine metabolism. Their respective ribonucleosides are xanthosine and inosine (see Figures 6 and 8). There are also extraordinary metabolites like 8-oxo-deoxyguanosine that are damaged, potentially harmful, non-canonical purines.

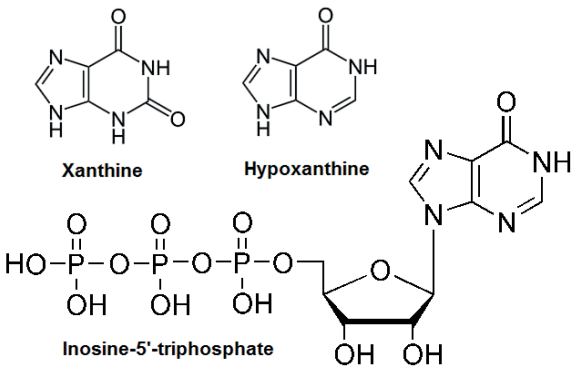


Figure 8. Chemical structures of the non-canonical purine nucleobases Xanthine and Hypoxanthine and the nucleotide Inosine 5'-triphosphate.

The non-canonical nucleotides are formed when canonical nucleotides are damaged by oxidative stress and deamination.[39-41] While the role of the canonical adenine and guanine based nucleotides is extensively studied, the potential role of the non-canonical purine based nucleotides xanthosine and inosine in the human metabolism has not yet been fully clarified and may be both beneficial and harmful. Inosine, inosine 5'-triphosphate (ITP) and to a lesser extend also inosine 5'-diphosphate (IDP) and IMP were found to have anti-inflammatory effects.[42-44] Cyclic ITP (cITP) may function as a second messenger.[45] On the other hand, incorporation of deaminated nucleotides like deoxy-Inosine 5`-triphospate (dITP) and deoxy-Xanthosine 5`-triphosphate (dXTP) into DNA and RNA is thought to have mutagenic consequences.[46,47] Multiple enzymes have been described that clear the cellular nucleotide pool from these potential harmful non-canonical nucleotides.[48] One of those enzymes is Inosine 5'-triphosphate pyrophosphohydrolase (Inosine triphosphatase or ITPase), which dephosphorylates ITP to IMP and xanthosine 5`-triphosphate (XTP) to xanthosine 5`-monophosphate (XMP). IMP and XMP are further processed to the canonical nucleotides AMP and GMP as described above (Figures 5 and 6).

INOSINE TRIPHOSPHATE PYROPHOSPHOHYDROLASE (ITPASE)

Substrates, expression and function

In 1964 Liakopoulou and Alivisatos were the first to describe this enzyme in human erythrocytes, found it to be very specific for ITP[49] and called it ITP phosphohydrolase (ITPase). Their initial conclusion that ITPase hydrolysed ITP to IDP and further to IMP was refuted by Vanderheiden, who showed in his experiments that IDP was not involved in the reaction and that ITPase *pyro*phosphohydrolysed ITP to IMP.[50] The natural substrates for ITPase are ITP, dITP and XTP.[51] Potentially the enzyme is somewhat promiscuous, for (d)GTP, (d)ATP, (d)CTP, TTP and UTP were found in some assays to be pyrophosphohydrolysed by ITPase, although 10-100 fold less efficient.[51] It was shown that (d)IMP and (d)IDP are no substrates at all, and that IDP is in fact an inhibitor of ITPase.[52] The specificity of ITPase for (d)ITP and XTP is unusually high compared to other enzymes of the nucleotide metabolism[53] and this is probably due to the structure of the enzyme (Figure 9).[54] ITPase consists of two monomers of 21.5 kDa, composed of 194 amino acids, forming a dimer. Each monomer consists of a long central β-sheet forming the floor of the active site, with an upper and a lower lobe, between which the substrate binds. The substrate specificity for ITP and XTP is explained by the hydrogen bonds they can form with the active site, while ATP and GTP have amino-groups in those binding locations, which do not allow hydrogen bonding.[55] Because neither of the ribose hydroxyl groups make strong contacts with the protein, ITPase can utilize both ITP as well as dITP.[55]

ITPase is not only expressed in erythrocytes but in a wide range of human tissues (from leukocytes, bone marrow and lymph nodes to solid organs, skeletal muscle, spinal cord and reproductive organs).[51,52] Both the specificity of the enzyme for (d)ITP and XTP and the fact that ITPase is more or less ubiquitously expressed in human tissues are consistent with the hypothesis that ITPase cleans the nucleotide pool from potential harmful nucleotides. Indeed, in embryos of mice with no ITPase activity, eight times higher levels of deoxy Inosine were found in the nuclear DNA compared to control mice embryos. Furthermore, the embryonic fibroblasts of these ITPase devoid mice embryos showed increased chromosome aberrations and accumulation of single-strand breaks in the nuclear DNA.[46]

Genetic basis of ITPase deficiency

In 1969 Vanderheiden measured elevated levels of ITP in the erythrocytes of different families. He suggested that these high levels of ITP were due to ITPase deficiency and that this deficiency was an inheritable trait.[56] In 1980, analysis of a de novo translocation between chromosomes X and 20 of a 13-year-old female showed that the gene *ITPA* encoding for ITPase was appointed to the short arm of chromosome 20.[57] However, it was not until 2001 that the *ITPA* gene was cloned and characterized.[51] One year later five single nucleotide

polymorphisms (SNPs) in this gene were identified. Three silent SNPs (138G>A, 561G>A and 708G>A) and two SNPs that lead to decreased ITPase activity: 94C>A (p.Pro32Thr, rs1127354) and 124+21A>C (or IVS2+21A>C, rs7270101). The 94C>A missense mutation leads to a substitution of proline to threonine amino acid at codon 32. This substitution disrupts the protein structure (Figure 9) and also leads to missplicing of exons 2 and 3.[58,59] Only homozygotes for the 94C>A SNP have nearly complete ITPase deficiency in erythrocytes, in the 94C>A heterozygotes 23% of the ITPase activity remains.[58] The 124+21A>C SNP leads to a less severe state of ITPase deficiency, by miss-splicing exon 3 only,[59] resulting in 60% of mean ITPase activity in heterozygotes and 30% in homozygotes.[58,60] The distribution of the *ITPA* SNPs varies within the different world populations. The 94C>A mutation is highest in Asian populations (14-19%) compared to Caucasian/African populations (6-7%) and Central/South American (1-2%).[61] The 124+21A>C SNP is extremely rare in Asian populations,[62,63] but more frequent in Caucasian populations (11-13%).[58,64,65] Since then, more SNPs have been identified, with varying degrees in residual ITPase activity.[60,62,66]

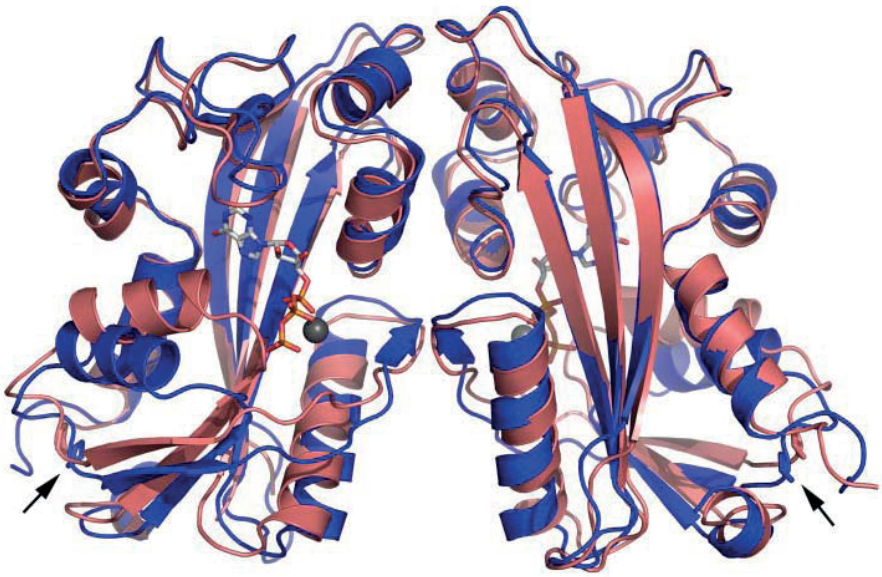


Figure 9. Structure of the Inosine triphosphate pyrophosphohydrolase enzyme. In blue: the unbound (apo) structure, in pink: the ITP-bound structure. The site of the SNP 94C>A (p.Pro32Thr, rs1127354) which leads to a decreased activity of the enzyme is indicated by an arrow for both monomers. *Adapted from: Stenmark, P. et al. Crystal structure of human inosine triphophatase. Substrate binding and implication of the inosine triphosphatase deficiency mutation P32T. The Journal of Biological Chemistry 2006, 282(5): 3182-3187.*

Phenotypic correlation of ITPase deficiency with human diseases

Over the years, multiple studies have been done to find phenotypic abnormalities in individuals with SNPs in the *ITPA* genotype or a decreased ITPase activity. The first to report an increased incidence (16%) of 'High ITP' in a mentally retarded population versus a population with normal intelligence (3%), was Fraser[67] in 1975. Additionally, in chronic paranoid schizophrenic patients a significant decrease in ITPase activity was found compared to a non-psychiatric control population.[68] In 2015 the association between Early Infantile Encephalopathy was found in 7 patients with several pathogenic mutations in *ITPA* that led to a severe degree of ITPase deficiency not restricted to erythrocytes.[66] And recently, in 2018, two families with a very distinctive clinical presentation of lethal infantile-onset dilated cardiomyopathy and the rare Martsolf syndrome, which is characterized by congenital cataracts, postnatal microcephaly, developmental delay, hypotonia and short stature, were found to have mutations in the *ITPA* gene leading to undetectable ITPase protein.[69]

A connection between *ITPA* mutations and malignancy has been hypothesized, although studies investigating this subject are still sparse. In one study an increase in mitochondrial DNA mutations was found in adult hematology patients (consisting of myelodysplastic syndrome, acute myeloid leukemia and chronic lymphocytic leukemia patients), carrying the 94C>A SNP, compared to patients carrying wild-type *ITPA*.[70] In another study *ITPA* is mentioned as one of the genes mutated in adenocarcinoma of the pancreas.[71]

One study reported on an unusually high prevalence of decreased ITPase activity and *ITPA* genotypes with SNPs in patients with pulmonary Langerhans' cell histiocytosis compared to a reference population (50% versus 11% respectively).[72]

THE ROLE OF *ITPA* SNPs IN DRUG METABOLISM

ITPA SNPs have been found to influence drug metabolism of azathioprine and its active metabolite mercaptopurine, methotrexate and ribavirin. Azathioprine is used as an immunosuppressive drug prescribed after organ transplantation, and for multiple autoimmune diseases, like inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus. Azathioprine is metabolized to mercaptopurine (6-MP) which is then further metabolized by multiple enzymatic steps of the purine salvage and interconversion pathways, including ITPase. In 2004, an association between adverse drug reactions of azathioprine such as influenza-like symptoms, rash and pancreatitis was described for the *ITPA* SNP 94C>A in a cohort of patients treated for inflammatory bowel disease.[73] However, these findings could not be confirmed in another study.[74] Since then, some studies reported an association between the 94C>A SNP and bone marrow depression such as decreased

leukocytes[75-77] or agranulocytosis,[78] flu-like symptoms[79] or arthralgia,[80] but in other studies no association could be demonstrated[81-84] in patients with inflammatory bowel disease. Also the outcome of azathioprine therapy was not clearly associated with *ITPA* SNPs.[80,85] SNP 124+21A>C was in all the studies but one[86] not associated with adverse events.[75,77,80,81,83,84] In patients with systemic lupus erythematosus (SLE) 94C>A was associated with a better response to low-dose azathioprine therapy,[87,88] but in patients with renal or liver transplants studies were inconclusive.[89-91] Taken together, in adult patients treated with azathioprine, the SNP 94C>A is associated with adverse events, although not undisputed.

In children treated with mercaptopurine (6-MP) for acute lymphoblastic leukemia the SNP 94C>A in the *ITPA* gene was associated with hepatic toxicity,[92-94] decreased leukocytes[94-96] and decreased event-free survival,[97] although again, these findings were not undisputed, as another study found the opposite effect on event-free survival in a comparable group of patients.[98] In patients treated with methotrexate for rheumatoid arthritis the SNP 94C>A a possible association with a worse clinical response was found.[99,100]

ITPA, ITPASE AND INFECTIOUS DISEASES

Hepatitis C

In patients treated with PEG-Interferon gamma and ribavirin for a hepatitis C virus (HCV) infection, the effect of SNPs in the *ITPA* gene on the occurrence of anemia and hemoglobin (Hb) decline is extensively studied.[101-107] In a meta-analysis by Pineda-Tenor in 2015,[108] the SNPs 94C>A, 124+21A>C and rs6051702 were all associated with protection against Hb decline. Furthermore, 94C>A was also significantly associated with protection against the occurrence of severe anemia and the necessity for ribavirin dose reduction during therapy. None of these studies investigated the association between ITPase activity and the occurrence of anemia and the degree of Hb decline.

HIV

In spite of a genotype distribution comparable to a non-HIV infected population, ITPase activity was found to be significantly decreased in erythrocytes of HIV-infected patients compared to a non-HIV infected control population.[64] However, no further studies were done to investigate whether this affected the occurrence of adverse events during the use of cART. For replication, HIV depends on the nucleotide metabolism of the infected host. For instance, HIV uses reverse transcriptase to convert its single-stranded RNA into double-stranded DNA, using the nucleotides available in the human cell, and further transcribes new RNA from this DNA after incorporation into the human genome, using the human nucleotide mechanism. The NRTIs are analogues of the natural ribonucleotides and

compete with these natural ribonucleotides for incorporation in the growing RNA chain. When incorporated, further RNA synthesis is stopped (chain termination) because of the NRTIs miss a 3'-hydroxyl group on the deoxyribose. To further improve HIV therapy by decreasing adverse events, more insight into the association between *ITPA* genotypes and ITPase activity on the one hand and adverse events during use of cART on the other hand could be important. Anti-retroviral drugs mimicking all nucleobases exist, but since ITPase is an enzyme in the human purine metabolism, we concentrated on the drugs abacavir (a guanosine analogue) and didanosine and tenofovir (adenosine analogues).

OUTLINE OF THE THESIS

The general aim of this thesis is to investigate the influence of the *ITPA* genetic SNPs 94C>A and 124+21A>C and the activity of the enzyme ITPase on the occurrence of adverse events during treatment for the infectious diseases HCV and HIV. While *ITPA* SNPs were previously found to be protective against hemolytic anemia during therapy with ribavirin for HCV, it is not clear to what extent other SNPs contribute to ITPase activity. Therefore, in **Chapter 2**, ITPase activity is compared with *ITPA* genotype for prediction of anemia during ribavirin use for HCV.

In HIV-infected patients the ITPase activity in erythrocytes was found to be decreased compared to a population not infected with HIV. As leukocytes are the main target cells for HIV-infection, in **Chapter 3** the expression of the ITPase protein in leukocytes and leukocyte subpopulations is explored in association with *ITPA* genotype. The results in HIV-infected patients are compared to a non-HIV infected population.

As the current therapy for HIV is highly effective, the main obstacle for treatment nowadays is adverse events during cART. In **Chapter 4**, we investigate the association of ITPase activity and *ITPA* genotype with the occurrence of adverse events during combination antiretroviral therapy for HIV. One of the most important adverse drug events during tenofovir use is nephrotoxicity. In order to test if ITPase activity and *ITPA* genotype could be used as a biomarker to predict nephrotoxicity during tenofovir use for HIV,these parameters are determined and compared in HIV-infected patients with and without nephrotoxicity, in **Chapter 5**.

Other important aspects brought on by the current effective HIV therapy, are long term consequences of cART and increasing diseases of older age, one of them being cardiovascular diseases. The association of ITPase activity and *ITPA* genotype with the occurrence

of cardiovascular diseases and metabolic events during cART for HIV-infection is further explored in **Chapter 6.**

To further unravel effects caused by HIV-infection itself and effects caused by cART, in **Chapter 7**, basic cell metabolomics of untreated HIV-infected patients are compared to a control population (the effect of HIV-infection). In addition metabolomics data prior to start of cART were compared to 12 months of therapy successfully suppressing HIV replication (the effect of cART).

In **Chapter 8** the results of these studies are discussed with respect to future perspectives.

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Chapter 2.

Inosine triphosphate pyrophosphohydrolase activity: more accurate predictor for ribavirin-induced anemia in hepatitis C infected patients than *ITPA* genotype.

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ABSTRACT

Background

ITPA polymorphisms have been associated with protection against ribavirin-induced anemia in chronic hepatitis C (HCV) patients. Here we determined the association of inosine 5'-triphosphate pyrophosphohydrolase (inosine triphosphatase or ITPase) enzyme activity with *ITPA* genotype in predicting ribavirin-induced anemia.

Methods

In a cohort of 106 HCV patients, hemoglobin (Hb) values were evaluated after 4 weeks (T_4) and at the time of lowest Hb value (T_{nadir}). ITPase activity was measured and *ITPA* genotype determined. Single-nucleotide polymorphisms (SNPs) tested were c.124+21A>C and c.94C>A. ITPase activity ≥1.11 mU/mol Hb was considered as normal.

Results

After 4 weeks of treatment, 78% of the patients with normal ITPase activity were anemic and 21% of the patients with low ITPase activity ($p<0.001$). Stratified by genotype, the percentages of anemic patients were: wt/wt 76%, wt/c.124+21A>C 46% ($p=0.068$), and wt/c.94C>A 29% ($p=0.021$). At T_{nadir} virtually all patients with normal ITPase activity were anemic, compared to only 64% of the patients with low activity ($p=0.02$). Thirteen patients had wt/c.124+21A>C genotype. Within this group all five patients with normal ITPase activity and only four of eight with decreased activity developed anemia. Presence of HCV RNA did not influence ITPase activity.

Conclusions

This study is the first to report that ITPase activity predicts the development of anemia during ribavirin treatment. ITPase activity and *ITPA* genotype have high positive predictive values for development of ribavirin-induced anemia at any time during treatment, but ITPase activity predicts ribavirin-induced anemia more accurately.

INTRODUCTION

The prevalence of hepatitis C (HCV) infection is estimated at approximately 2.2%–3% of the world population (130– 170 million people).[1] The life expectancy of infected patients is reduced significantly because of high risks for liver cirrhosis and hepatocellular carcinoma.[2] HCV therefore is one of the main reasons for liver transplantation in Europe and the US.[3,4] In order to prevent these complications, patients with HCV infection have been treated with the combination of pegylated-interferon-α plus ribavirin,[5-7] in later years combined with protease inhibitors, such as telaprevir[8] or boceprevir[9] and since recently simeprevir[10] or the nucleoside polymerase inhibitor sofosbuvir.[11] Response to anti-HCV therapy is influenced by both viral and host factors as well as drug toxicity.[12] Viral genotype has been a strong predictor for treatment response. While viral remission is around 70%–80% in patients infected with HCV genotypes 2 and 3, only 50%–60% of the patients with HCV genotypes 1 and 4 acquire a sustained virological response (SVR).[13] Host factors contributing to therapeutic outcome have been identified as single-nucleotide polymorphisms (SNPs) in the *IL28B* and *LDLR* genes.[14-18] An important and common adverse drug reaction limiting optimal HCV therapy is ribavirin-induced anemia. It has been demonstrated that two functional *ITPA* SNPs, rs1127354 (c.94C>A) and rs7270101 (c.124+21A>C), are associated with protection from ribavirin-induced anemia.[19-25]

In recent years, the biological and pharmacogenetic significance of *ITPA* and its corresponding enzyme inosine 5'-triphosphate pyrophosphohydrolase (inosine triphosphatase or ITPase) have become a focal point of research, bringing many interesting and surprising data. Complete ITPase deficiency is strictly confined to erythrocytes and is considered to be a benign condition. No primary, causal, clinical symptoms are known under normal circumstances. However, ITPase activity lowering SNPs in *ITPA* are associated with adverse drug reactions to the thiopurines azathioprine and 6-mercaptopurine. This association is still subject of a lively discussion.[26-30] The pharmacogenetic significance of *ITPA* appeared not to be limited to the thiopurines, but may also be of significance for the purine analog ribavirin. In our present study we demonstrate that ITPase activity seems a more accurate predictor for ribavirin-induced anemia than *ITPA* genotype.

MATERIALS AND METHODS

Patients

Consecutive HCV infected patients attending the outpatient clinic of the Erasmus Medical Center in Rotterdam, The Netherlands were included during 6 months with the aim of inclusion of 100 patients. The following data were collected: gender, age, hemoglobin (Hb),

white blood cell count (WBC), HCV genotype and HCV RNA in serum, type of medication, start and end of treatment, and treatment outcome. Treatment was given according to the Dutch national guidelines at that time[31] with peginterferon-α-2a or peginterferon-α-2b (in a dosage of 180 μg or 1.5 μg/kg once a week), in combination with ribavirin 800–1200 mg a day depending on patient weight. One patient also received boceprevir and one patient also received miravirsen in addition to the ribavirin and peginterferon-α.

Plasma HCV-RNA levels were measured with the use of the COBAS AmpliPrep/COBAS TaqMan HCV assay, version 1.0 (Roche molecular systems) from October 2008 until March 2012, version 2.0 (Roche molecular systems) was used from March 2012 until June 2012. Anemia was defined as Hb reduction of ≥1.9 mmol/L compared to pre-treatment values and/or Hb concentrations <7.5 mmol/L for females and <8.0 mmol/L for males.[22,32]

The study was performed according to the Helsinki Declaration and approved by the Medical Ethical Committees of the Erasmus Medical Center, Rotterdam, The Netherlands. The participants provided their written informed consent to participate in this study.

ITPase activity

ITPase activity was determined as described previously with some minor modifications[33] and measured by formation of inosine 5'-monophosphate (IMP) from ITP. Briefly, 3 μL whole blood was incubated with 2.00 mM ITP, 50 mM $MgCl_2$, 0.5 mM Dithothreitol (DTT) and 0.2 mM α-,β-methyleneadenosine 5'-diphosphate (AMP- CP) in 75 mM Tris in a final volume of 200 μL. All chemicals were of the highest grade and purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Samples were prepared and analyzed in duplicate. In addition, blanks (negative controls) and pool samples (positive controls) were also analyzed for ITPase activity to confirm correct sample preparation, analysis and quality control. High performance liquid chromatography (HPLC) separations were performed on a Supelcosil LC-18 S column (Sigma-Aldrich, Zwijndrecht), using an Alliance separation system (Waters, Etten-Leur, The Netherlands) coupled to a Jasco multi-wavelength detector (Jasco Benelux, IJsselstein, The Netherlands). Data were analyzed with TotalChrom data acquisition and handling software (Perkin-Elmer, Groningen, The Netherlands). ITPase activity was expressed as milliUnits of IMP formed from ITP per mol hemoglobin (mU/mol Hb). The intra-assay variation coefficient was <5%, and the inter-assay variation coefficient was <10%. The cut-off value discriminating between normal or decreased ITPase activity was set at 1.11 mU/mol Hb (=4 mmol IMP/mmol Hb/h), which is the lowest value within the 95% CI for *ITPA* wild type (wt/wt) carriers.[34,35]

ITPA genotype analysis

Genomic DNA was isolated from whole blood using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) and genotyped for two *ITPA* polymorphisms; wt/c.94C>A (p.Pro32Thr, rs1127354) and wt/c.124+21A>C (rs7270101). When no polymorphisms were detected at both positions and the ITPase activity was within the wt/wt reference intervals, the genotype was considered to be wt/wt. M13-tagged primers forward 5'-TGTAAAACGACGGCCAGTCTTAGGAGATGGGCAGCAG and 5'-CAGGAAACAGCTATGACCCACAGAAAGTCAGGTCACAGG reverse were used in a PCR reaction, which consisted of 1xAmplitaq Gold Mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 8% glycerol, and 200 nM of each primer. PCR conditions were 40 cycles with an annealing temperature of 60°C. The resulting 241 bp PCR product was purified and directly sequenced in both directions using the Big Dye Terminator kit and was subsequently analyzed on an ABI 3720 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The resulting sequence was aligned with *ITPA* reference sequence NM_033453.2. All sequences were evaluated by two independent laboratory experts.

Statistical analysis

Results were analyzed using GraphPad Prism 5.01 (Graphpad Software for Windows, San Diego, CA, USA), Microsoft Excel (Microsoft, Redmond, WA, USA) software and IBM SPSS Statistics 20 (IBM Corporation, New York, NY, USA) software. Pearson's χ^2-tests, Fisher's exact tests (in the case of small sample sizes) or T-tests for independent samples were used to determine significant differences. p-Values <0.05 were considered to be statistically significant.

RESULTS

Patient characteristics

A total of 106 HCV infected patients in various stages of infection were included (Table 1). In our cohort there was a male predominance and HCV genotype 1 was most prominent. In total 69 patients were treated for chronic HCV infection and SVR was reached in 30 (43.5%) of the patients. The most prominent *ITPA* genotype was wt/wt (68.9%). The occurrence of wt/c.124+21A>C and wt/c.94C>A *ITPA* genotype variants were 19.8% and 9.4%, respectively. One patient was homozygous for c.124+21A>C and one was compound heterozygous, i.e. c.124+21A>C/c.94C>A. Our cohort showed expected allele frequencies for both loci and did not differ from the reference population.[35,36] The population was in Hardy-Weinberg equilibrium. Age, pre-treatment Hb levels and white blood cell counts were not significantly different between patients with different *ITPA* genotypes. As expected, mean ITPase activity correlated with *ITPA* genotype (Table 1).

Table 1. Characteristics of the study population

Characteristic	**Total Population (n=106)**	**Treated Population (n=69)**
Age, median years (min-max)		
Total	51 (20-88)	52 (20-79)
Wt/wt	51 (20-88)	52 (20-79)
Wt/c.124+21A>C	51 (33-76)	52 (39-76)
Wt/c.94C>A	55 (41-65)	54 (41-59)
Gender, n (%)		
Male	68 (64.2)	51 (73.9)
Female	38 (35.8)	18 (26.1)
HCV genotype, n (%)		
Genotype 1	63 (59.4)	44 (63.8)
Genotype 2/3	28 (26.4)	20 (28.9)
Genotype 4	8 (7.5)	5 (7.2)
Unknown	7 (6.6)	-
***ITPA* genotype,** n (%)		
Wt/wt	73 (68.9)	49 (71.0)
Wt/c.124+21A>C	21 (19.8)	13 (18.8)
Wt/c.94C>A	10 (9.4)	7 (10.1)
Other	2 (1.8)	-
HCV genotype 1, n (%)		
Total	63 (59.4)	44 (63.8)
Wt/wt	45 (71.4)	33 (75.0)
Wt/c.124+21A>C	11 (17.5)	7 (15.9)
Wt/c.94C>A	7 (11.1)	4 (9.1)
ITPase mean activity[a] ± SD		
Wt/wt	1.64 ± 0.47	1.67 ± 0.50
Wt/c.124+21A>C	1.01 ± 0.29	1.03 ± 0.37
Wt/c.94C>A	0.46 ± 0.16	0.50 ± 0.17
Other	0.33 ± 0.19	-
Absolute ITPase activity (%)		
Wt/wt	100%	
Wt/c.124+21A>C	62%	
Wt/c.94C>A	28%	
Other	20%	
ITPase mean activity[a] ± SD		
HCV RNA <5 copies/ml	1.44 ± 0.69	
HCV RNA ≥ 5 copies/ml	1.34 ± 0.53	
ITPase activity[a]**,** n (%)		
< 1.11	29 (27.4)	16 (23.2)
≥ 1.11	77 (72.6)	53 (76.8)

Table 1. Characteristics of the study population (continued)

Characteristic	Total Population (n=106)	Treated Population (n=69)
Mean white blood cell count[b] ± SD	5.9 ± 2.4	5.5 ± 2.4
Mean Hb pre-treatment[c] ± SD		9.0 ± 1.0
Treatment outcome, n (%)		
SVR[d]		30 (43.5)
Relapse		22 (31.9)
Non-response / termination		17 (24.6)
Spontaneous clearance	7 (6.6)	

[a] mU/mol Hb; [b] $x10^9$/L; [c] mmol/L; [d] SVR, Sustained virological response

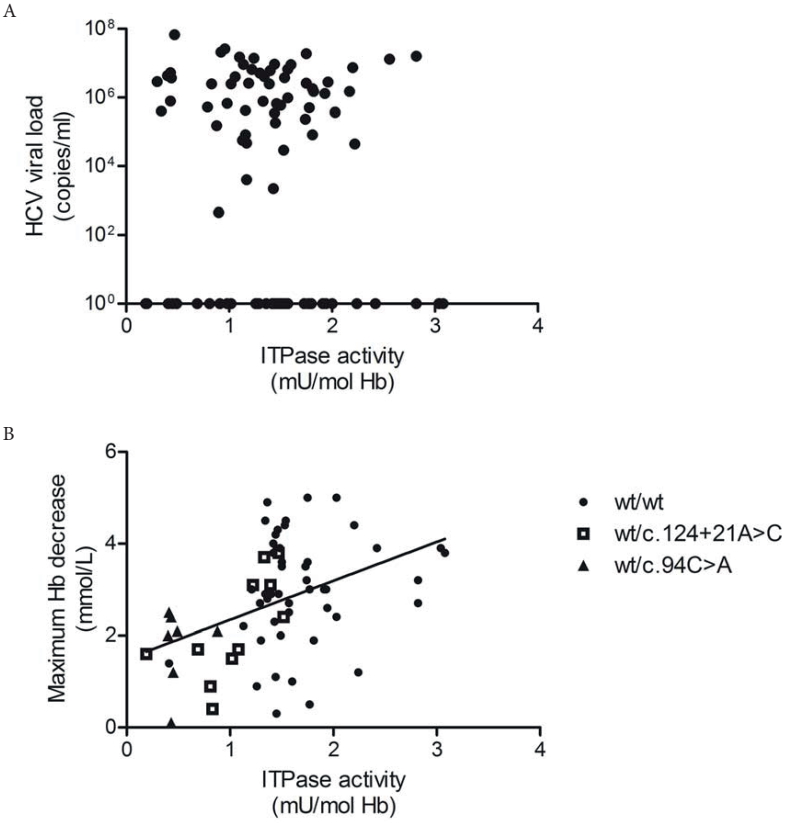

Figure 1: (A) ITPase activity and HCV viral load. Plasma HCV viral load (copies/ml) is plotted against ITPase activity (milliUnits IMP/mol Hb). Neither the presence nor the level of plasma HCV viral load is correlated to ITPase activity. HCV, hepatitis C virus; ITPase, inosine triphosphate pyrophosphohydrolase. (B) Association of ITPase activity and Hb decrease. ITPase activity (milliUnits IMP/mol Hb) is plotted against maximum Hb decrease during therapy. *ITPA* genotypes are displayed in different shapes (circles: wt/wt, squares: wt/c.124+21A>C, triangles: wt/c.94C>A). Higher ITPase activity is associated with increased Hb decline. ITPase, inosine triphosphate pyrophosphohydrolase; Hb, Hemoglobin.

Hepatitis C and ITPase activity

ITPase activity was not statistically significantly different in HCV-infected patients and non-HCV controls (data not shown).[35] Presence or absence of HCV-RNA was not associated with ITPase activity (Figure 1A), regardless of genotype. In patients with a detectable HCV-RNA, the viral load was not associated with ITPase activity (Figure 1A).

Hemoglobin levels and ITPase activity

In total 53 of 77 patients with normal ITPase activity, and 16 of 29 patients with decreased ITPase activity were treated with pegylated-interferon-α plus ribavirin. ITPase activity was significantly associated with Hb decrease (Table 2, Figure 1B, p<0.001). Of the patients having normal ITPase activity, 78.0% (n=39) were anemic after 4 weeks of therapy (T_4), compared to 21.4% (n=3) of the patients with reduced ITPase activity, see Table 2 (p<0.001). Exactly 92.2% of patients with normal ITPase activity developed anemia at any moment during therapy (T_{nadir}), compared to 64.3% of patients with low ITPase activity (p=0.02, Table 2). Test characteristics are shown in Table 3. The positive predictive value (PPV) of normal ITPase activity was 78% for anemia at 4 weeks and 92% for the development of anemia at any time during therapy. The negative predictive value (NPV) of a decreased ITPase activity for the development of anemia was 79% and 36%, respectively, for T_4 and T_{nadir}.

Table 2. Comparison of ITPase activity and *ITPA* genotype in development of anemia, and percentage of successful treatment outcome (SVR) in the treated population (n=69).

	ITPase activity (mU/mol Hb)			***ITPA* genotype**				
	≥ 1.11 (n=53)	**< 1.11 (n=16)**	**p-value**	**Wt/wt (n=49)**	**Wt/c.124+21A>C (n=13)**	**p-value**	**Wt/c.94C>A (n=7)**	**p-value**
Pre-treatment								
Hb[a]	9.0 ± 1.0[b]	8.9 ± 1.0	0.84	9.0 ± 1.0[b]	8.9 ± 0.7	0.84	9.3 ± 1.3	0.45
T_4								
Hb[a]	7.3 ± 1.0[c]	8.7 ± 1.0[b]	<0.001	7.3 ± 1.0[c]	7.9 ± 1.2[b]	0.06	8.8 ± 1.4	0.001
Anemia; n (%)	39 (78.0)[c]	3 (21.4)[b]	<0.001	35 (76.1)[c]	5 (45.5)[b]	0.07	2 (28.6)	0.02
Reduction[a]	1.7 ± 1.1[c]	0.4 ± 0.4[b]	<0.001	1.7 ± 1.1[c]	1.1 ± 1.3[b]	0.17	0.5 ± 0.4	0.006
T_{nadir}								
Hb[a]	5.9 ±1.1[b]	7.5 ±1.2[b]	<0.001	6.0 ± 1.1[b]	6.9 ± 1.1[b]	0.02	7.5 ± 1.5	0.002
Anemia; n (%)	47 (92.2)[b]	9 (64.3)[b]	0.02	43 (91.5)[b]	9 (81.8)[b]	0.32	4 (57.1)	0.04
Reduction[a]	3.1 ± 1.1[b]	1.5 ± 0.7[b]	<0.001	3.0 ± 1.2[b]	2.2 ± 1.1[b]	0.04	1.8 ± 0.9	0.012
SVR; n (%)	23 (43.4)	7 (43.8)	0.98	22 (44.9)	5 (38.5)	0.68	3 (42.9)	0.99

[a]Mean ± SD (mmol/L); [b] Values missing from 2 patients; [c] Values missing from 3 patients

Hemoglobin levels and *ITPA* genotype

Treatment with pegylated-interferon-α plus ribavirin was started in 49 of 73 patients with the *ITPA* wt/wt genotype, in 13 of 21 patients with *ITPA* wt/c.124+21A>C genotype and

Table 3. Comparison of ITPase activity and *ITPA* genotype in occurrence of anemia after 4 weeks of therapy (T_4) and at any time during therapy (T_{nadir}) and positive (PPV) and negative (NPV) predicting test characteristics.

	T_4 (n=64)			T_{nadir} (n=65)		
	Anemia	**No anemia**	**Predictive value**	**Anemia**	**No anemia**	**Predictive value**
ITPase activity[a]						
≥1.11	39	11	PPV: 78%	47	4	PPV: 92%
<1.11	3	11	NPV: 79%	9	5	NPV: 36%
***ITPA* genotype**						
Wt/wt	35	11	PPV: 76%	43	4	PPV: 91%
Wt/c.124+21A>C+Wt/c.94C>A	7	11	NPV: 61%	13	5	NVP: 28%

[a] mU/mol Hb

in 7 of 10 patients with *ITPA* wt/c.94C>A genotype. At Week 4 of therapy, anemia was observed in 76.1% of the patients carrying *ITPA* wt/wt, 45.5% of the patients carrying *ITPA* wt/c.124+21A>C (p=0.07) and 28.6% of the patients with *ITPA* wt/c.94C>A genotype (Table 2, p=0.02).

Anemia at any time during treatment (T_{nadir}) occurred significantly less frequently in patients with the wt/c.94C>A genotype (57.1%) compared to patients with the wt/wt genotype (91.5%) (p=0.04, Table 2). Hb at T_{nadir} was significantly higher in patients with the wt/c.124+21A>C *ITPA* genotype, and Hb reduction was significantly less compared to wt/wt *ITPA* genotype, but there was no difference in frequency of anemia (p=0.32).

ITPase activity vs. *ITPA* genotype

Of the patients with the wt/c.124+21A>C genotype, 38.5% (5 of 13) had a normal ITPase activity (Table 4). Of these five patients, four developed anemia at T_4 (80%), whereas in the eight patients with the same genotype, but with decreased ITPase activity, only one patient became anemic. At T_{nadir} all wt/c.124+21A>C patients with normal ITPase activity developed anemia in contrast to only four of the eight patients with decreased ITPase activity.

In all patients carrying wt/c.94C>A *ITPA* genotype ITPase activity was decreased and anemia was present in four of seven patients. Of the wt/wt genotype carrying patients, 48 of 49 had a normal ITPase activity and the patient with low ITPase activity developed anemia.

PPV for wt/wt genotype and ITPase activity were not different for both T_4 and T_{nadir} (Table 3). NPV for the ITPase activity lowering *ITPA* genotypes (wt/c.124+21A>C and wt/c.94C>A together) was lower compared to NPV for ITPase activity <1.11 mU/mol Hb for both T_4 (61% vs. 79%) and T_{nadir} (28% vs. 36%).

Table 4. Occurrence of anemia in treated patients according to *ITPA* genotype and ITPase activity after 4 weeks of therapy (T_4) and at any time during therapy (T_{nadir}).

***ITPA* genotype**	**Total, n**	**T_4, n (%)**	**T_{nadir}, n (%)**
Wt/wt	49		
Activity[a] <1.11	1		
No anemia		1 (100)	
Anemia			1 (100)
Activity[a] ≥1.11	48		
No anemia		10 (21)	4 (8)
Anemia		35 (73)	42 (88)
Unknown		3 (6)	2 (4)
Wt/c.124+21A>C	13		
Activity[a] <1.11	8		
No anemia		5 (63)	2 (25)
Anemia		1 (13)	4 (50)
Unknown		2 (25)	2 (25)
Activity[a] ≥1.11	5		
No anemia		1 (20)	
Anemia		4 (80)	5 (100)
Wt/c.94C>A	7		
Activity[a] <1.11	7		
No anemia		5 (71)	3 (43)
Anemia		2 (29)	4 (57)
Activity[a] ≥1.11	0		
No anemia			
Anemia			

[a] mU/mol Hb

Treatment outcome

SVR was achieved in 43.5% of the patients (n=30), whereas in 56.5% (n=39) treatment failed due to relapse, serological non-response, or termination of therapy because of adverse events. SVR was not associated with ITPase activity or *ITPA* genotype (Table 2), nor with anemia (data not shown). Table 5 shows the treatment outcome stratified by HCV genotype. Taking into account HCV genotype, still SVR was not associated with ITPase activity (Figure 2A) or *ITPA* genotype (Figure 2B). Also per-protocol analysis of the patients adherent to the entire treatment regimen, did not show an association between SVR and ITPase activity or *ITPA* genotype (data not shown).

Table 5. Treatment outcome per hepatitis C genotype, ITPase activity and *ITPA* genotype.

HCV genotype	Treatment outcome	ITPase activity[a]		*ITPA* genotype		
		≥ 1.11 (n=53)	< 1.11 (n=16)	Wt/wt (n=49)	Wt/c.124+21A>C (n=13)	Wt/c.94C>A (n=7)
1						
	SVR[b]	11	3	11	2	1
	Non-response	6	3	5	2	2
	Relapse	16	2	14	3	1
	Termination	3	0	3	0	0
2/3						
	SVR[b]	11	4	10	3	2
	Non-response	1	0	1	0	0
	Relapse	0	2	0	1	1
	Termination	0	2	0	2	0
4						
	SVR[b]	1	-	1	-	-
	Non-response	2	-	2	-	-
	Relapse	2	-	2	-	-
	Termination	0	-	0	-	-

[a] mU/mol Hb; [b] SVR, sustained virological response

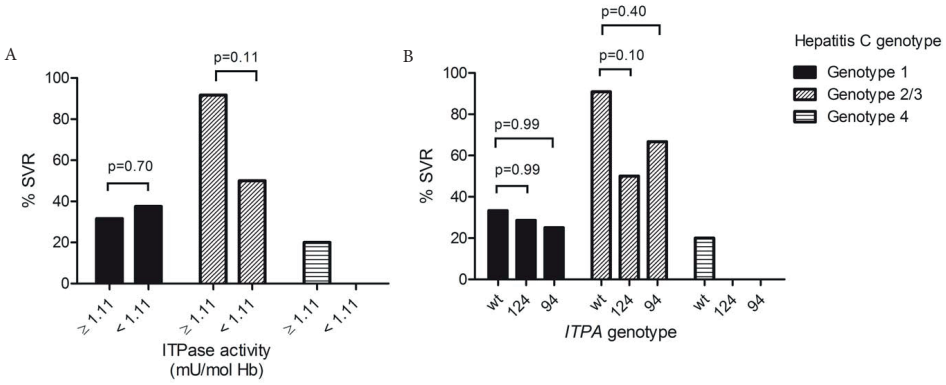

Figure 2. (A) The percentages of patients reaching SVR are shown when patients are stratified by hepatitis C genotype. No significant differences were observed between patients with ITPase activity ≥1.11 mU/mol Hb and with patients with ITPase activity <1.11 mU/mol Hb. SVR, sustained virological response; ITPase, inosine triphosphate pyrophosphohydrolase. (B) The percentages of patients reaching SVR are shown when patients are stratified by hepatitis C genotype. No significant differences were observed between patients with *ITPA* genotype wt/wt and patients with *ITPA* genotypes wt/c.124+21A>C and wt/c.94C>A. SVR, sustained virological response; wt, wt/wt; 124, wt/c.124+21A>C; 94,wt/c.94C>A.

DISCUSSION

This is, to our knowledge, the first study evaluating the association of ITPase enzyme activity and ribavirin-induced anemia and comparing it to *ITPA* genotype in patients treated for chronic HCV. All studies evaluating the association of *ITPA* polymorphisms with ribavirin-induced anemia assumed that in HCV patients, the reported ITPase activity directly corresponds to a specific *ITPA* polymorphism.[19,20,22,23,37] Here we show that an *ITPA* variant such as wt/c.124+21A>C leads to a variety of ITPase activities ranging from as low as 0.19 to as high as 1.52 mU/mol Hb and association is less direct as has previously been assumed. More in depth analysis showed that negative predicting value for ribavirin-associated anemia of the wt/c.124+21A>C genotype was only 18%. Within this group, all the patients with normal ITPase activity developed anemia throughout the treatment period, compared to 50% of the patients with decreased ITPase activity.

Most studies[20,22,25,37,38] only investigated Hb values after 4 weeks of therapy as at this time point many patients may start with erythropoietin treatment to stimulate red blood cell production. In our cohort, mean time to nadir was 4 months with 61% of patients having anemia at T_4, and 84% having anemia at T_{nadir}. ITPase activity was statistically significantly associated with anemia at both T_4 and T_{nadir}.

In two studies *ITPA* genotype polymorphisms were found to be protective for anemia during the course of the entire treatment.[39,40] The predictive value of *ITPA* genotype was similar to that reported in the literature in our hands, despite the small sample size.[22,24,37] Differences in occurrence of anemia were only statistically significant for *ITPA* wt/wt compared to wt/c.94C>A.

No influence of HCV presence or titer could be detected, this is in contrast with our observation in human immunodeficiency virus (HIV)-infected population, in which the geno-phenotype correlation differs significantly from the reference population.[35] Similar to *ITPA* polymorphisms in other studies, ITPase activity was not predictive for SVR in our cohort.[19,21,37] Some studies report higher SVR rates for patients with ITPase activity decreasing *ITPA* genotypes,[25,41] and a recent study reported reduced relapse risk following treatment for HCV genotype 2/3 in these genotypes.[42] However, probably due to small sample size, we were not able to confirm these findings in our study. Addition of the new protease inhibitors telaprevir or boceprevir improves response rates to 70% in patients with genotype 1.[8,9] Although the influence of protease inhibitors and the nucleoside polymerase inhibitor sofosbuvir on ITPase activity needs to be established, ribavirin is still a part of these treatment regimens and it might be cost saving to prevent adverse events like severe anemia by more tailor-made treatment.

Other purines are still widely used in the treatment of other diseases (i.e., abacavir and tenofovir in HIV, azathioprine and 6-mercaptopurine in inflammatory bowel disease and acute lymphoblastic leukemia) and may be also influenced by ITPase activity. So even though ribavirin is becoming less important in the treatment of HCV infection, further research to the impact of *ITPA* genotype and ITPase activity on the degradation of purine analogs will still be important.

Despite the fact that ITPase activity was measured in whole blood, the activities measured correlated with the genotype-specific reference values established in erythrocytes in our laboratory and by others.[35,43,44] Although markedly more men were included in this study, this did not influence the assessment of anemia, as gender-specific Hb reference values were used.

It is not clear why decreased ITPase activity protects from ribavirin-induced anemia. A direct association between ribavirin levels, ITPase activity and anemia has been hypothesized but could not be proven.[37] Although it has been reported that ITPase deficiency decreased the need for ribavirin dose reduction,[40] this could not be confirmed in HCV mono-infected patients[19] nor in HIV/HCV co-infected patients.[45] Thus, direct influence of ITPase activity on ribavirin levels does not seem to be a plausible explanation.

Another possible explanation for the assumed protective effect of *ITPA* SNPs has been suggested by Hitomi et al.,[46] who stated that *ITPA* polymorphisms resulted in decreased ITPase activity causing accumulation of ITP. However, ITP was found to only accumulate in the erythrocytes of patients homozygous for c.94C>A *ITPA* genotype variant.[44,47] Furthermore, Hitomi et al.[46] stated that ITP was a substitute for GTP in the generation of AMP by adenylosuccinate synthetase (ADSS). If this were to be correct, the proposed protective effect could only be effective in erythrocytes of patients who are homozygous for c.94C>A. However, neither Hitomi and coworkers, nor any other author (including our group) has, to the best of our knowledge, demonstrated ADSS activity in erythrocytes. The exact mechanism is still not elucidated and needs a more mechanistic approach.

In conclusion, this study is the first to describe the direct correlation of ITPase activity and decrease in Hb values during treatment with ribavirin. In addition, we demonstrated that ITPase activity is a better pre-treatment parameter to predict ribavirin-induced anemia than *ITPA* genotype.

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Chapter 3.

Inosine triphosphate pyrophosphohydrolase expression: decreased in leukocytes of HIV-infected patients using combination antiretroviral therapy.

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ABSTRACT

Objective

In HIV-infected patients, the enzyme Inosine triphosphate pyrophosphohydrolase (ITPase), involved in purine nucleotide homeostasis, was found to be decreased in erythrocytes. Since purine analogues are pivotal in the HIV treatment, a better understanding of ITPase expression in $CD4^+$ lymphocytes may lead to better understanding of nucleotide metabolism and (adverse) effects.

Design

Cross-sectional, cohort, observational study.

Methods

HIV-infected and control patients above 18 years were included. All DNA samples were genotyped for the 2 functional *ITPA* SNPs; c.94C>A (rs1127354) and c.124+21A>C (rs7270101). ITPase expression was determined by flow cytometry in all leukocyte subsets.

Results

Fifty-nine HIV-infected patients and 50 controls were included. Leukocyte subtype distribution showed no difference in monocytes and granulocytes, but lymphocytes were higher in HIV-infected patients ($p<0.001$). ITPase expression was highest in activated monocytes and lowest in lymphocytes. In HIV-infected patients, the percentage of ITPase positive cells was less in all leukocyte and lymphocyte subsets compared with controls ($p<0.01$). In HIV-infected patients, 97.4% of $CD4^+$ lymphocytes were ITPase positive versus 99.9% in controls ($p=0.002$) and 85.9% versus 99.6% of $CD8^+$ lymphocytes ($p<0.0001$), respectively. Stratification according to genotype revealed no significant differences in ITPase expression in leukocytes in HIV-infected and control patients.

Conclusions

HIV-infection seems to be interfering with the nucleotide metabolism in leukocytes, including $CD4^+$ lymphocytes, by decreasing ITPase expression, independently of *ITPA* genotype. Given that active metabolites of purine-analogue reverse transcriptase inhibitors are potential substrates for ITPase, these results warrant further research towards effectiveness and adverse events of purine analogues and ITPase activity.

INTRODUCTION

The Human Immunodeficiency Virus (HIV) is a retrovirus that copies its single-stranded RNA into double-stranded DNA, which is incorporated into the DNA of the host. Conversely, new viral RNA is transcribed from the infected host DNA and packaged into new virus particles. These processes are entirely dependent on human nucleotide metabolism in the host lymphocytes. This makes nucleotide metabolism both a target and a vehicle for anti-viral therapy, reflected by the numerous anti-retroviral nucleoside analogues that have been created. Interestingly, there are indications that nucleotide metabolism is impaired in lymphocytes and erythrocytes of HIV-infected individuals. HIV-1 positive lymphocytes were demonstrated to be unable to expand their ribonucleotide pool (both purines and pyrimidines) to the same extent as HIV-1 negative lymphocytes after a strong mitogenic stimulation, prohibiting DNA-replication and leading to cell death.[1] Furthermore, the activities of thymidine kinase 1 and thymidylate kinase (TMP-kinase), both enzymes in the pyrimidine nucleotide biosynthetic pathway, were significantly decreased in lymphocytes of HIV-infected patients compared with controls.[2,3] Purine nucleotide homeostasis may be compromised, as the enzyme Inosine 5'-triphosphate pyrophosphohydrolase (Inosine triphosphatase; ITPase) was found to be significantly decreased in erythrocytes of HIV-infected patients compared with a control population.[4] Since antiviral purine analogues are pivotal in the treatment of HIV-infection, a better understanding of ITPase expression in lymphocytes may lead to a better understanding of nucleotide metabolism and the effects of HIV treatment.

ITPase, encoded by the polymorphic gene *ITPA* (OMIM #147520), is one of the scavenger enzymes eliminating the potentially cyto- or genotoxic noncanonical nucleoside triphosphates from the nucleotide pool.[5] Multiple single nucleotide polymorphisms (SNPs) of *ITPA* have been described,[6] among which 2 SNPs are proven to decrease ITPase activity: c.94C>A (p.Pro32Thr, NCBI rs1127354) and c.124+21A>C (NCBI rs7270101). The SNP c.94C>A is found in most world populations, albeit with varying frequency,[7] whereas the SNP c.124+21A>C has not been found or was extremely rare among Asian populations.[8–10] *ITPA* genotype[6,11–13] and decreased ITPase activity[14] have been associated with protection against anemia in the treatment of hepatitis C with the purine analogue ribavirin. Furthermore, although conflicting reports exist,[15,16] SNPs in the *ITPA* genotype have been associated with adverse drug events during thiopurine therapy with azathioprine and 6-mercaptopurine.[17,18] Also, the SNP c.94C>A led to higher concentrations of methyl-mercaptopurine nucleotides in patients treated for acute lymphoblastic leukemia and a higher probability of severe febrile neutropenia in these patients, when mercaptopurine dose had been adjusted for *TPMT* (thiopurine S-methyltransferase) genotype.[19] Rare truncating mutations in the *ITPA* gene have recently been associated with a severe early-infantile encephalopathy.[20] In an *ITPA*

knockout mouse model, ITPase deficient mice showed features of growth retardation and cardiac myofiber disarray and died soon after birth.[21]

In HIV-infected patients, the decreased erythrocyte ITPase activity was independent of the presence of SNPs in the *ITPA* gene, as it was seen in both wild-type and SNP carriers.[4] This finding raised the question whether in leukocytes as well, ITPase activity is decreased, and thus purine metabolism is altered, independent of *ITPA* genotype in HIV infected patients on cART. We therefore analyzed the presence of the ITPase protein in leukocytes and leukocyte subpopulations, in association with *ITPA* genotype in a HIV-infected and a control population.

METHODS

Patients

HIV-seropositive patients, visiting the HIV outpatient clinic of the Maastricht University Medical Centre (MUMC+) aged 18 years and older, were eligible for inclusion in the study. Consecutive patients were asked for participation and included after written informed consent was obtained. Data regarding age, gender at birth, country of birth, race, CD4 nadir, and date of start combination Antiretroviral therapy (cART), were obtained from the Dutch HIV monitoring foundation (Stichting HIV Monitoring; SHM). The control population consisted of anonymous samples from the general hospital population of the Zuyderland Medical Center in Heerlen. All control samples were used according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies. The study was approved by the Medical Ethics Committee of the MUMC+ and all patients signed written informed consent.

Flow cytometric ITPase expression analysis

ITPase expression was determined by flow cytometry as described earlier.[22] In short, EDTA anticoagulated peripheral blood samples were processed and analyzed within 24 hours after collection. One hundred microliters of whole blood was incubated with a cocktail of directly conjugated antibodies.[22] Cell suspensions were incubated for 15 minutes in the dark at room temperature (RT). A Fix&Perm strategy (GAS004; Caltag, Invitrogen, Carlsbad, CA) was performed after washing with phosphate-buffered saline. The fixation step consisted of the addition of 100 mL fixation reagent for 15 minutes at RT and 2 wash steps (phosphate-buffered saline). This was followed by the addition of the permeabilization medium and anti-ITPase MoAb (clone 2H8, isotype IgG2a, catalog number H00003704-M01, Abnova, Taipei City, Taiwan). Except some isoforms of ITPase protein, no other human proteins have a similar sequence, thus the specificity of the antibodies used to detect the ITPase protein is considered

to be high and the chance we measured other protein products than the ITPase protein seems negligible. After incubation for 15 minutes at RT and one wash step, 5 mL PE-conjugated anti-IgG2a (Southern Biotech, Birmingham, AL) was added. Finally, after incubation (15 minutes at RT) and washing, 500 mL CellWash (BD) was added. Samples were analyzed with a BD FACSCanto (BD Biosciences, San Jose, CA). Cell-bound fluorescent labels were excited with a 488 nm, air-cooled, solid-state argon ion laser, followed by a 17 mW 633 nm, Helium–Neon laser. The flow cytometer was set up daily using 7-color beads (BD) with automatic compensation settings using the FACSCanto software to achieve optimal instrument performance. Sample analysis was completed when 5000 lymphocytes were collected. All measured events were stored and analyzed. The fluorescence signal data were recorded with logarithmical amplification. FACSDiva software (BD) was used for the analysis of acquired data. A negative control (Fluorescence-minus-one: no anti-ITPase but with addition of PE-conjugated anti-IgG2A) was used to set a threshold at a maximum of 5% positivity to exclude auto-fluorescence and to confirm ITPase-specific labeling. The expression of ITPase was determined by determining the median fluorescent intensity of a certain leukocyte population.

Gating strategy

Parental gating, based on scatter signals and immuno-fluorescence staining as described earlier,[22] resulted in a step-wise separation of subpopulations of leukocytes with accumulated complexity of phenotypical characteristics.

DNA isolation and *ITPA* genotyping

Total DNA was extracted from blood using the Blood L Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and the Hamilton Microlab STAR Line (Hamilton, Bonaduz, Switzerland). Specific intronic primers with an additional M13-tag were used to amplify exon 2 of the *ITPA* gene and flanking intronic regions (*ITPA*_ex2F-CTTTAGGAGATGGGCAGCAG; *ITPA*_ex2R-CACAGAAAGTCAGGTCACAGG). Amplification was performed in a 10 mL reaction volume using Amplitaq Gold 360 Master Mix (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) with 5% 360 GC Enhancer, 2 pmol of each primer, and 10 ng DNA. The cycle conditions were 96°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds with a final elongation step of 72°C for 10 minutes. The resulting 245-bp PCR product was bi-directionally sequenced using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI3730XL genetic analyzer (Applied Biosystems, Foster City, CA). The 2 common functional polymorphisms c.94C>A (p.Pro32Thr; NCBI rs1127354) and c.124+21A>C (NCBI rs7270101) were determined using Mutation Surveyor DNA variant analysis software (SoftGenetics, State College, PA) with genomic NCBI reference sequence NC_000020. All sequences were evaluated independently by 2 laboratory experts.

Statistical analysis

Results were analyzed using GraphPad Prism 5.01 (Graphpad Software for Windows, San Diego, CA), Microsoft Excel (Microsoft, Redmond, WA) software, and IBM SPSS Statistics 20 (IBM Corporation, New York, NY) software. Independent samples 2-tailed T-test was used to determine significant differences. P-values <0.05 were considered to be statistically significant.

RESULTS

Patient characteristics

A total of 59 HIV-infected patients and 50 controls were included (Table 1). The mean age did not differ significantly between the groups. There was a male predominance in the HIV-infected patients. All but 1 patient used cART and the mean CD4 nadir of this group was 206 $CD4^+$ lymphocytes per liter. Although all mean values of the hematological parameters assessed were within the reference values, significant differences between the HIV-infected patients and the control patients were found (Table 1). Mean corpuscular volume and mean corpuscular hemoglobin levels were higher in the HIV-infected population, whereas hematocrit and mean corpuscular hemoglobin concentration was lower. The HIV-infected patients had a significantly lower white blood cell and platelet count compared with the control population.

Leukocytes and leukocyte subsets

The leukocyte subtype distribution showed no difference in percentages of monocytes (6.7% versus 6.7%; p=0.9, respectively) and granulocytes (56.7% versus 61.1%; p=0.08, respectively) between HIV-infected and control patients (data not shown). The HIV-infected patients had a statistically significant higher percentage of lymphocytes (29.4% versus 23.1%; p<0.001, respectively). No difference was found in the size of the fraction of B-lymphocytes between HIV-infected patients and controls (9.80% versus 10.03%; p=0.80, respectively). The percentage of T-lymphocytes was significantly higher in HIV-infected patients (80.0% versus 74.0%; p=0.001, respectively), with decreased T-helper cells ($CD4^+$ T-cells) (37.6% versus 61.6%; p<0.0001) and increased cytotoxic T-cells ($CD8^+$ T-cells) (57.9% versus 32.6%; p<0.0001) compared with the control population. The percentage of Natural killer cells was significantly lower in HIV-infected patients (10.2% versus 16.6%; p< 0.0001).

Presence of ITPase in leukocyte subsets

High percentages of ITPase positive cells could be demonstrated in the control population; lymphocytes 99.5%, monocytes 98.8%, and granulocytes 95.3% were ITPase positive. Less lymphocytes (90.9%) and granulocytes (77.1%) were ITPase positive in HIV-infected

Table 1. Demographic, clinical and hematological characteristics of patients and controls.

Characteristic	HIV-infected (N=59)	Controls (N=50)	Reference values, range	p-value
Age, Median years (range)	49.6 (28.3-68.2)	56.8 (15.9-90.2)		
Male gender, n (%)	45 (76.3)	50 (50.0)		
***ITPA* genotype**, n (%)				
Wt/wt	40 (67.8)	26 (52.0)		
Wt/c.124+21A>C	7 (11.9)	12 (24.0)		
Wt/c.94C>A	7 (11.9)	9 (18.0)		
c.124+21A>C/c.124+21A>C	1 (1.7)	3 (6.0)		
c.94C>A/c.124+21A>C	2 (3.4)	-		
Unknown	2 (3.4)	-		
Mean CD4 nadir ± SD	205.8 ± 152.4			
cART	58 (98.3)			
Race, n (%)				
Caucasian	47 (79.7)			
Hispanic	1 (1.7)			
African	4 (6.8)			
Asian	5 (8.5)			
Mix	2 (3.4)			
Mean WBC, $x10^9$/L ± SD	6.33 ± 2.05	8.69 ± 2.82	4.00–10.00	<0.001
Mean RBC, $x10^{12}$/L ± SD	4.67 ± 0.66	4.77 ± 0.34	4.20–6.20	0.31
Mean Hb, mmol/L ± SD	10.67 ± 9.40	8.94 ± 0.70	7.50–11.00	0.20
Mean Ht, L/L ± SD	0.48 ± 0.06	0.43 ± 0.03	0.36–0.51	<0.001
Mean MCV, fL ± SD	102.76 ± 9.69	90.24 ± 3.66	80.00–100.00	<0.001
Mean MCH, ± SD	2.03 ± 0.22	1.87 ± 0.09	1.70–2.10	<0.001
Mean MCHC, g/dl ± SD	19.80 ± 1.02	20.78 ± 0.61	19.30–22.50	<0.001
Mean Platelets, $x10^{12}$/L ± SD	222.07 ± 68.47	248.48 ± 60.62	130.00–400.00	0.04

cART, combined Antiretroviral therapy; WBC, White blood cells; RBC, Red blood cells; Hb, Hemoglobin; Ht, Hematocrit; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration

patients (Figure 1A) compared with the control population (p<0.0001). Furthermore, all lymphocyte subtypes showed statistically significant lower percentages of ITPase positive cells in HIV-infected patients (Figure 1B). In the controls, all lymphocyte subtypes had a mean percentages of ITPase positive cells of 99% or more, whereas in HIV-infected patients 91% of T-lymphocytes, 97% of B-lymphocytes, and 87% of natural killer cells were ITPase positive (p<0.0001 for all). Although the CD4/CD8 ratio of T-lymphocytes is decreased in HIV-infected patients, both CD4 lymphocytes and CD8 lymphocytes had less ITPase positive cells compared with the control patients; 97.4% versus 99.9% for $CD4^+$ lymphocytes (p=0.002) cells and 85.9% versus 99.6% for $CD8^+$ lymphocytes (p<0.0001).

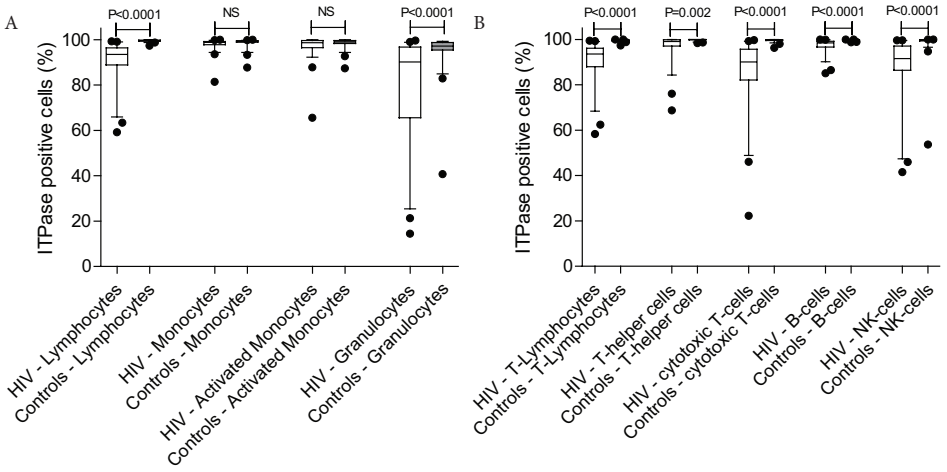

Figure 1: Comparison of percentages of ITPase positive cells in (A) white blood cell subtypes and (B) lymphocyte subsets between HIV-infected patients (white bars) and control patients (gray bars). Displayed are: the median, the 25-75% percentiles (bars), upper and lower 1,5 times interquartile range (whiskers) and outliers (dots). Except for (activated) monocytes, all the compared cell types showed significant decreases of ITPase positive cells in HIV-infected patients (p=0.002 in T-helper cells, p<0.0001 in all others).

Quantitation of ITPase expression

ITPase expression was determined in the ITPase positive cell fraction. ITPase expression (expressed as median fluorescent intensity) was highest in activated monocytes and lowest in lymphocytes, in both HIV-infected patients and controls regardless of HIV-infection (Figure 2A). In HIV-infected patients, ITPase expression was lower in (activated) monocytes, granulocytes, and lymphocytes compared with controls (Figure 2A, p<0.0001 for all). In all lymphocyte subsets, ITPase expression was significantly lower in HIV-infected patients compared with controls (Figure 2B, p-values <0.0001 for all).

Association of ITPase expression in leukocytes with *ITPA* genotype

ITPA allele frequencies did not differ significantly between HIV-infected patients and the control group (Table 1). Wt/wt was the most prominent *ITPA* genotype (67.8% in HIV-infected and 52% in controls). The ITPase expression was not associated with *ITPA* genotype (comparison between wt/wt, wt/c.124+21A>C and wt/c.94C>A + other) in monocytes, lymphocytes, and granulocytes (Fig. 3).

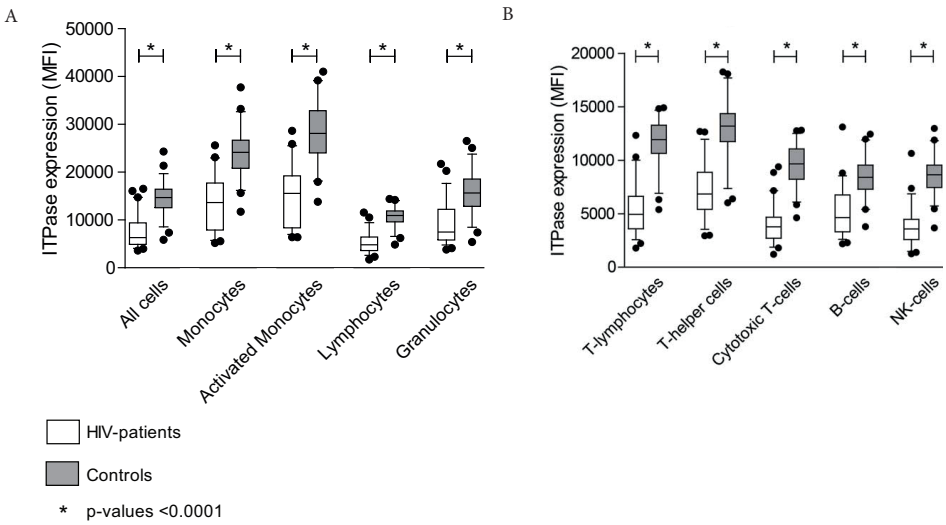

Figure 2: Comparison of ITPase expression (expressed as Median fluorescent intensity; MFI) in white blood cell subtypes (A) and lymphocyte subsets (B) between HIV-infected patients (white bars) and control patients (gray bars). Displayed are: the median, the 25-75% percentiles (bars), upper and lower 1,5 times interquartile range (whiskers), outliers (dots). All the compared cell types showed significant decreases of MFI in HIV-infected patients ($p<0.0001$ for all).

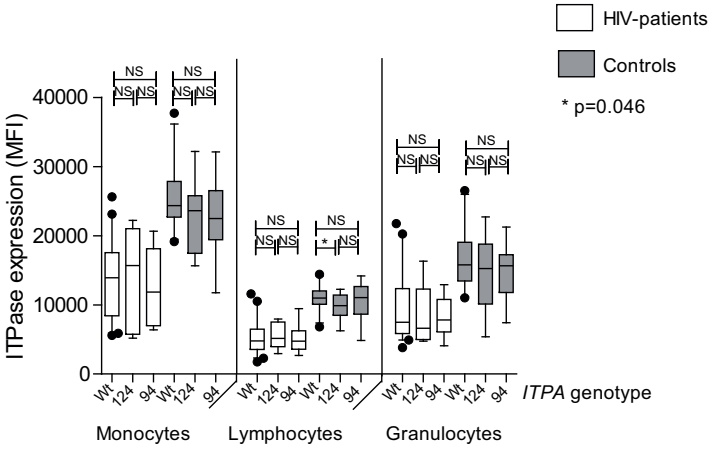

Figure 3. Stratification of different leukocyte subtypes by *ITPA* genotype and the corresponding ITPase expression (expressed as Median fluorescent intensity, MFI). No association was found between *ITPA* genotype (wt=wt/wt; 124=wt/c.124+21A>C; 94=wt/c.94C>A) and ITPase expression in HIV-infected patients (white bars) and control patients (gray bars) in monocytes, lymphocytes and granulocytes (no significant differences except for the comparison between wt/wt and wt/c.124+21A>C in lymphocytes of control patients; p=0.046).

DISCUSSION

Here, we report a striking significant decrease in ITPase expression in leukocyte subtypes and lymphocyte subsets in HIV-infected patients compared with controls. We observed an overall decrease in ITPase expression in white blood cells of treated HIV-positive patients, regardless of genotype suggesting that the decrease in ITPase expression in leukocytes was because of HIV-infection or cART, albeit that the influence of nucleoside analogues is likely to be limited.[4]

Regardless of HIV status, ITPase protein expression was highest in monocytes, being especially high in activated monocytes. This may reflect the phagocytic activity, as the destruction of pathogens and infected cells either by oxygen dependent and independent pathways releases nucleotides and very likely also noncanonical nucleotides, such as Inosine triphosphate (ITP).

Competition for the intracellular nucleotide pools between the host white blood cells and HIV appears to be a major contributor to HIV pathogenesis. Earlier reports showed that pyrimidine nucleotide metabolism (both de novo and salvage) is severely impaired in HIV-infected lymphocytes.[1–3] After mitogenic stimulation, HIV-infected lymphocytes were unable to extend both their purine and pyrimidine nucleotide pools to allow proliferation. A major difference between HIV-infected lymphocytes and controls was that the former rely predominantly on purine salvage metabolism to meet the cellular requirement opposed to de novo synthesis of purine nucleotides being the predominant biosynthetic route in the latter. Sterile alpha motif and histidine-aspartic domain-containing protein 1 (SAMHD1) is a human defense protein that inhibits the HIV-1 replication by depleting intracellular deoxynucleotide pools.[23] In HIV-2 infection, this action is counteracted by the virion-packaged accessory protein.[23] These findings suggest that the infected lymphocyte's ultimate defense mechanism is to cause a fatal imbalance in the intracellular nucleotide pool to prevent HIV-integration and replication. Our observation of reduced ITPase expression in HIV-infected lymphocytes is in line with this hypothesis. Decreased activity of ITPase leads to decreased scavenging of noncanonical nucleotides, including ITP and deoxy-ITP which contributes to the intracellular nucleotide imbalance in HIV-infected lymphocytes by increasing the availability of non-canonical nucleotides for DNA and RNA replication, causing erroneous incorporation into nucleic acids, potentially leading to programmed cell death and mutagenesis.[24]

The decreased ITPase expression and thus possibly the decreased ITPase activity in the blood compartment[4] may have implications for the efficacy of purine analogue reverse transcriptase inhibitors. It can be expected that the efficacy of nucleos(t)ide reverse tran-

scriptase inhibitors (NRTIs) of which the nucleotide triphosphate form exerts the virostatic effect is enhanced, whereas the efficacy of NRTIs of which the nucleotide monophosphate exerts the virostatic effect is reduced. This implies treatment with purine NRTIs tenofovir, didanosine, and abacavir, which are active in their nucleotide triphosphate form, benefit from the observed decreased ITPase expression. This is in contrast to the earlier reported decreased phosphorylation of ziduvodine in HIV-infected lymphocytes due to decreased thymidine kinase 2 activity.[2]

ITPase protein and *ITPA* mRNA are likely to be ubiquitously expressed in all nucleated cells.[25–27] With respect to erythrocytes, a clear-cut genotype–phenotype correlation exists, both in HIV-infected patients and in controls.[28,29] According to our present findings this seems not to be the case in white blood cells, neither in HIV-infected patients and controls. Our previous results in erythrocytes may suggest decreased stability of the P32T ITPase protein and the wild type protein in the erythrocytes of HIV-infected individuals. Because no protein synthesis takes place in erythrocytes, a HIV-induced instability or reduced biosynthesis of the ITPase protein is readily detected. A possible explanation for the fact that this is not observed in our present work may be that in white blood cells *ITPA* is transcribed and the ITPase protein is synthesized continuously, despite the amount of mRNA encoding the entire protein being reduced due to altered splicing of the pre-mRNA in the *ITPA* 94C>A and 121+21A>C.[30] Although ITPase expression appears to be generally decreased in blood, there may still exist pharmaco-genetic phenomena associated with *ITPA* genotype, apart from the present observation.

In conclusion, this study is the first to report that the expression of ITPase is significantly decreased in lymphocytes and granulocytes of treated HIV-infected patients, compared with a control population. Given that purine NRTIs are active in their triphosphate form, the present results underline their importance in the backbone of cART. Our findings corroborate previous findings that control over intracellular nucleotide pools is vital for both HIV and its host for survival.

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Chapter 4.

Erythrocyte Inosine triphosphatase activity: a potential biomarker for adverse events during combination antiretroviral therapy for HIV.

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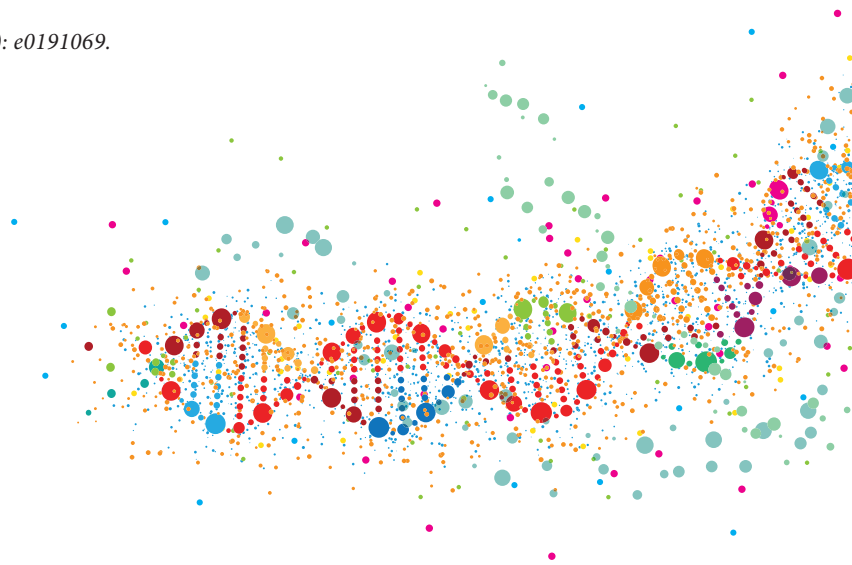

ABSTRACT

The purine analogues tenofovir and abacavir are precursors of potential substrates for the enzyme Inosine 5'-triphosphate pyrophosphohydrolase (ITPase). Here, we investigated the association of ITPase activity and *ITPA* genotype with the occurrence of adverse events (AEs) during combination antiretroviral therapy (cART) for human immunodeficiency virus (HIV) infection. In 393 adult HIV-seropositive patients, AEs were defined as events that led to stop of cART regimen. ITPase activity ≥ 4 mmol IMP/mmol Hb/hour was considered as normal. *ITPA* genotype was determined by testing two *ITPA* polymorphisms: c.94C>A (p. Pro32Thr, rs1127354) and c.124+21A>C (rs7270101). Logistic regression analysis determined odds ratios for developing AEs. In tenofovir-containing regimens decreased ITPase activity was associated with less AEs (p=0.01) and longer regimen duration (p=0.001). In contrast, in abacavir-containing regimens decreased ITPase activity was associated with more AEs (crude p=0.02) and increased switching of medication due to AEs (p=0.03). *ITPA* genotype wt/wt was significantly associated with an increase in the occurrence of AEs in tenofovir-containing regimens. Decreased ITPase activity seems to be protective against occurrence of AEs in tenofovir-containing cART, while it is associated with an increase in AEs in abacavir-containing regimens.

INTRODUCTION

Combination anti-retroviral therapy (cART) for patients infected with the human immunodeficiency virus (HIV) has been increasingly effective over the last years. However, adverse events (AEs) are still common and can be severe. Predicting whether AEs will occur with specific antiretroviral drugs would be a valuable tool in the choice of cART regimens. Although determination of HLA-B*57:01 status to predict hypersensitivity for abacavir, is widely used,[1] no other biomarkers or genetic susceptibility traits are known that can be used to predict AEs associated with cART.

The enzyme Inosine 5'-triphosphate pyrophosphohydrolase (ITPase) is an enzyme which converts inosine 5'-triphosphate (ITP) to inosine 5'-monophosphate (IMP) and xanthosine 5'-triphosphate (XTP) to xanthosine 5'-monophosphate (XMP). IMP and XMP are central metabolites in the purine metabolism, from which adenosine 5'-monophosphate (AMP) and guanosine 5'-monophosphate (GMP) and subsequently adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP) can be formed. ITPase is encoded by the *ITPA* gene located on chromosome 20p. *ITPA* is a polymorphic gene and a substantial part of the Western population carries one of the single nucleotide polymorphisms (SNPs) *ITPA* c.94C>A or *ITPA* c.124+21A>C.[2] The frequency of c.94C>A is highest in Asian populations (14–10%) compared to Central/South American (1–2%) and Caucasian and African populations (6–7%).[2] The SNP c.124+21A>C is extremely rare in Asian populations.[3-5] These polymorphisms result in a decreased ITPase activity.[6,7] Homozygosity for *ITPA* c.94 C>A leads to a null activity solely in erythrocytes, while activity in nucleated cells remains detectable.[8] True ITPase deficiency in humans is very rare and causes a severe form of early infantile encephalopathy.[9] Decreased ITPase activity and the frequent *ITPA* SNPs are associated with a reduced risk to develop ribavirin-induced hemolytic anemia in patients on treatment for hepatitis C,[10-12] and with an increased risk of AEs in patients treated with thiopurines.[13-15]

Our interest in the role of ITPase in the treatment of HIV-infected patients stems from the fact that both abacavir and tenofovir as well as didanosine are purine analogues and are therefore potential precursors of substrates for ITPase. Similarly to its association with AEs during the use of thiopurines and ribavirin, ITPase activity might influence the occurrence of AEs during purine analogues containing cART. Moreover, the majority of HIV-infected patients showed a decreased erythrocyte ITPase activity compared to healthy controls.[16] Therefore, we determined whether ITPase activity and *ITPA* genotype are associated with the occurrence of AEs during cART with purine analogues in a cohort of HIV-infected patients. In addition, we tested whether the active metabolites of abacavir and tenofovir (i.e. carbovir-triphosphate and tenofovir-diphosphate resp.) are substrates for the enzyme ITPase.

MATERIALS AND METHODS

Patients

Consecutive HIV-infected patients attending the outpatient clinic of the Maastricht University Medical Center in Maastricht, The Netherlands, between March first 2009 and January first 2014, who were treated with cART were included in this study after providing a written informed consent. Demographic, laboratory and clinical data from the moment the patients entered medical care for the HIV-infection until January first 2014 were retrieved from the database of the Dutch HIV monitoring foundation (Stichting HIV Monitoring; SHM), also known as the AIDS Therapy Evaluation in the Netherlands (ATHENA) and if needed from the medical records. The SHM includes data on demographics, comorbidities, cART, clinical, immunological and virological parameters of individuals in HIV care since January 1996 in any of the 26 HIV treatment centers in the Netherlands. Patients can opt-out after being informed by their treating physician of the purpose of collection of clinical data. The study was censored at January first 2014. The study was performed according to the Helsinki Declaration and approved by the Medical Ethical Committee of the Maastricht University Medical Center, Maastricht, The Netherlands.

ITPase activity

Erythrocyte ITPase activity was determined once per patient in the period of March first 2009 until January first 2014 and determined as described previously[17] and assessed by formation of inosine 5'-monophosphate (IMP) from inosine 5'-triphosphate (ITP). ITPase activity was expressed as mmoles of IMP formed from ITP in one hour per mmol hemoglobin (mmol IMP/mmol Hb/hour). The cut-off value discriminating between normal or decreased ITPase activity was set at 4 mmol IMP/mmol Hb/hour, which is the lowest value within the 95% CI for *ITPA* wt/wt carriers.[16,18]

In order to test whether or not carbovir-triphosphate and tenofovir-diphosphate are substrates for ITPase, the enzyme activity assay was carried out as described, using 1 millimolar of ITP, carbovir-triphosphate or tenofovir-diphosphate. Erythrocytes of a non-HIV infected confirmed wild type *ITPA* genotype carrier individual was used for this experiment. Experiments were performed in triplicate. Carbovir-triphosphate and tenofovir-diphosphate were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada).

ITPA genotype analysis

Genomic DNA was isolated from whole blood using the Wizard Genomic DNA purification kit (Promega, Madison, WI) and genotyped using sanger sequencing for the two *ITPA* polymorphisms; c.94C>A (p.Pro32Thr, rs1127354) and c.124+21A>C (rs7270101), as previously

described.[16] When both polymorphisms were not detected, the genotype was considered to be wild type (wt/wt). All sequences were evaluated by two independent laboratory experts.

Adverse events

For a uniform definition of AEs, AEs resulting in stopping or switching of the cART regimen and AEs that could be objectified in the laboratory were included. AEs were defined as stopping or switching for any reason except for the reasons virological failure, interaction with other medication, simplification or intensification of the regimen, drug taken off the market, patient deceased, low cART blood levels. Before statistical analyses, AEs were categorised and their potential association with ITPase activity or *ITPA* genotype was determined. The categorisation of the AEs was as follows: any AE (all categories of AE named hereafter taken together), gastro-intestinal (abdominal pain, nausea, diarrhoea, stomach ache, loss of appetite, pancreatitis), neurological (psychiatric complaints or dizziness, sleeping disorder, headache, tremor, disorders of taste), renal (renal insufficiency, kidney stones, nephritis, hypophosphatemia or lactate acidosis as reported reason for stopping the cART regimen or MDRD <60 ml/min/1.73 m^2 or phosphate <0.6 mmol/L in at least two separate measurements without other obvious cause), skin (rash or abscess at the site of injection) and liver related (liver failure as reported reason for stopping the cART regimen or alanine aminotransferase and/or aspartate aminotransferase >90 U/L without other obvious cause, in at least two separate measurements, or in one measurement in case of only two measurements performed during that regimen).

Statistical analysis

Results were analysed using IBM SPSS Statistics 21 (IBM Corporation, New York, USA) and SAS system for windows version 9.3. Pearson-chi-square-tests, Fisher's exact test and independent samples T-tests were used to determine significant differences. *P* values <0.05 were considered to be statistically significant. The occurrence of adverse events was analyzed with logistic regression with repeated statement and adjusted for cumulative total duration of cART therapy, cumulative duration of purine analogue therapy of all prior regimens and duration of the current regimen. Analysis included check for significant interaction with treatment and ITPase activity. When abacavir, tenofovir or didanosine were used concomitantly in one cART regimen, these regimens were excluded from the analysis.

RESULTS

Patient characteristics

Of 393 HIV infected patients, 205 (52.2%) patients had a decreased ITPase activity (Table 1). There were no statistically significant differences with respect to age, gender, ethnicity and alcohol use between the groups having normal and decreased ITPase activity. Mean CD4 nadir did not differ significantly between the group of patients with decreased ITPase activity (216 ± 161 cells/μL) and normal ITPase activity (200 ± 146 cells/μL).

cART regimens

In total 393 patients accounted for 1464 prescribed regimens. The median number of regimens per patient was 3, with a maximum of 18 regimens in one patient (Table 1). In total 38.992 person months of anti-retroviral therapy were observed. 9% of the regimens started before 1998. Purine analogues (tenofovir, abacavir and didanosine) were frequently prescribed (n=601, n=244 and n=128 respectively) and the proportion of purine analogue containing regimens (69.3%) did not differ between the group of patients with normal ITPase activity and the group of patients with decreased ITPase activity (p=0.09). 17 regimens (1.1%) contained both tenofovir and didanosine, 16 regimens (1.1%) contained abacavir and didanosine and 9 regimens (0.6%) contained tenofovir and abacavir. These regimens were excluded from further analysis, so 1422 regimens were used to assess the association of ITPase activity and *ITPA* genotype with AEs.

ITPase activity and *ITPA* genotype in HIV patients

ITPA genotype was determined in 386 patients. The most prominent *ITPA* genotype was wt/wt (265 patients, 67.4%). The occurrence of wt/c.124+21A>C and wt/c.94C>A *ITPA* genotype variants was 68 (17.3%) and 35 (8.9%) respectively. Other variants occurred in only 4.6% of the patients (homozygous c.124+21A>C n=5, homozygous c.94C>A n=2 and heterozygous c.124+21A>C/c.94C>A n=11). Mean ITPase activity correlated with *ITPA* genotype, with the highest ITPase activity in the wt/wt genotype. However, within the *ITPA* genotype wt/wt, 90 (34.0%) patients had decreased ITPase activity and within the *ITPA* genotype wt/c.124+-21A>C, 9 (13.2%) of the patients had normal ITPase activity. The other *ITPA* genotypes were only associated with a decreased ITPase activity.

Association of ITPase activity with AEs

734 regimens were prescribed in 205 patients with decreased ITPase activity and 688 in 188 patients with normal ITPase activity (N.S., Table 1). In 6.8% of the regimens the reason for switching or stopping cART regimen was unknown. In total, AEs were present during 714 regimens (50.2%) with 356 AEs in patients with decreased ITPase activity and 358 in

patients with normal ITPase activity (N.S.). The occurrence of AEs and the effect of the ITPase activity are displayed in Table 2 and Figure 1 respectively.

Table 1. Demographic and clinical characteristics of the patients (n=393) with ITPase activity <4 and ≥4 mmol IMP/mmol Hb/hour.

Characteristic	**ITPase activity**		**P-value**
	<4[a] (n=205)	**≥4**[a] (n=188)	
Age, median years (range)	50.6 (20-81)	49.7 (27-84)	0.80
Male Gender, n (%)	164 (80.0)	155 (82.4)	0.53
Race, n (%)			0.81
White non-Hispanic	164 (80.0)	147 (78.2)	
White Hispanic	5 (2.4)	4 (2.1)	
African	22 (10.7)	27 (14.4)	
Asian or other	14 (6.9)	10 (5.3)	
Mean ITPase activity ± SD	2.44 ± 1.12	5.24 ± 1.09	<0.001
***ITPA* genotype,** n (%)			<0.001
Wt/wt	90 (43.9)	175 (93.1)	
Wt/c.124+21A>C	59 (28.8)	9 (4.8)	
Wt/c.94C>A or other[b]	53 (25.9)	-	
Unknown	3 (1.5)	4 (2.1)	
Alcohol, n (%)			0.51
<2 IU/day	157 (76.6)	133 (70.7)	
≥2 IU/day	35 (17.1)	36 (19.1)	
Unknown	13 (6.3)	19 (10.1)	
Recreational drugs[c], n (%)			0.04
None	120 (58.5)	92 (48.9)	
Yes	50 (24.4)	55 (29.3)	
Unknown	35 (17.1)	41 (21.8)	
Hepatitis B co-infection, n (%)			0.25
No / cleared	182 (88.8)	167 (88.8)	
Yes	10 (4.9)	6 (3.2)	
Unknown	13 (6.3)	15 (8.0)	
Hepatitis C co-infection, n (%)			0.52
No	174 (84.9)	159 (84.6)	
Yes	27 (13.2)	22 (11.8)	
Unknown	4 (2.0)	7 (3.7)	
Mean CD4 nadir ± SD	215.6 ± 160.9	200.1 ± 145.7	0.32
Median year of start cART (range)	2006 (1987-2013)[d]	2006 (1987-2013)[e]	0.25
Start cART < the year 1998 (%)	9.7	8.3	0.68
Median number of cART regimens/patient (range)	3 (1-14)	3 (1-18)	0.74
Total number of cART regimens	734	688	

Table 1. Demographic and clinical characteristics of the patients (n=393) with ITPase activity <4 and ≥4 mmol IMP/mmol Hb/hour. (continued)

Characteristic	**ITPase activity**		**P-value**
	<4[a] (n=205)	**≥4**[a] (n=188)	
Median duration of cART regimen, months (range)	18.0 (0-161)[f]	15.5 (0-160)[g]	0.06
Purine containing cART, n (%)			0.09
Tenofovir	306 (40.1)	295 (41.1)	
Abacavir	131 (17.2)	113 (15.8)	
Didanosine	51 (6.7)	77 (10.7)	
Mean duration purine containing cART, months ± SD			
Tenofovir	29.2 ± 24.7	22.5 ± 22.0	0.001
Abacavir	34.9 ± 35.6	41.4 ± 40.8	0.19
Didanosine	24.7 ± 27.9	19.4 ± 22.4	0.24

[a] mmol IMP/mmol Hb/hour; [b] Other, homozygous c.124+21A>C or homozygous c.94C>A or heterozygous c.124+21A>C/c.94C>A; [c] heroin, cocaine, amphetamines, 3,4-methylenedioxymethamphetamine (MDMA), cannabis, lysergic acid diethylamide (LSD), ketamine, gamma-hydroxybutyric acid (GHB) and akylnitrate ('poppers'); [d] Missing in 1 patient; [e] Missing in 2 patients; [f] Missing in 1 patient; [g] Missing in 3 patients

Tenofovir

Normal ITPase activity was significantly associated with AEs in regimens containing tenofovir (167 vs 137 respectively, Odds ratio (OR) 0.65 for decreased ITPase activity versus normal activity; 95% CI 0.46–0.92; p=0.01), see Figure 1. Tenofovir-containing regimens used with normal ITPase activity were significantly more often switched because of AEs than for other reasons compared to regimens used with a decreased ITPase activity (91 of 281 vs 70 of 293 respectively (reason for switch unknown in 27 regimens), p=0.02). Furthermore, mean regimen duration was statistically significantly longer in patients with a decreased ITPase activity (29.2 vs 22.5 months; p=0.001), see Table 1.

Of all the renal AEs that occurred, 48.7% were in the group of regimens containing tenofovir (n=55) and of these 63.6% occurred in the patients with normal ITPase activity (p=0.04; OR 0.51; 95% CI 0.27–0.96 for patients with decreased ITPase activity versus normal activity), see Figure 2.

Abacavir

In regimens containing abacavir 61.2% of all AEs occurred in the patients with decreased ITPase activity and 38.8% in the patients with normal ITPase activity (crude p=0.02). After correction using logistic regression, OR remained elevated for decreased ITPase activity versus normal ITPase activity (1.69) but did not reach significance (p=0.08), see Figure 1. Significantly more often the cART regimen was switched because of AEs instead of other reasons when ITPase activity was decreased compared to normal ITPase activity (50 of 124

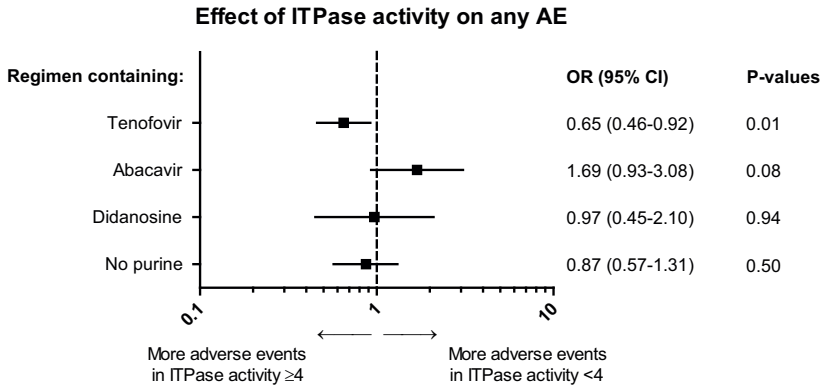

Figure 1. Effect ITPase activity on total adverse events. The effect of decreased versus normal ITPase activity on the occurrence of total adverse events (grouped by regimens containing tenofovir, abacavir or didanosine and regimens without tenofovir, abacavir or didanosine) is plotted. Odds ratio with 95% confidential interval and matching p-values are displayed. The analyses were conducted with repeated statement, adjusted for cumulative total duration of cART, cumulative duration of purine analogue therapy of all prior regimens and duration of the current regimen.

vs 29 of 108 respectively (reason for switch unknown in 12 regimens), p=0.03). In general, more adverse events tended to occur in patients with decreased ITPase activity, e.g. gastro-intestinal and skin related AEs (Figure 2). Of the cART regimens containing abacavir, 6.1% were stopped or changed because of skin related AEs and 73.3% of these events occurred in the patients with decreased ITPase activity, however this difference was not statistically significant.

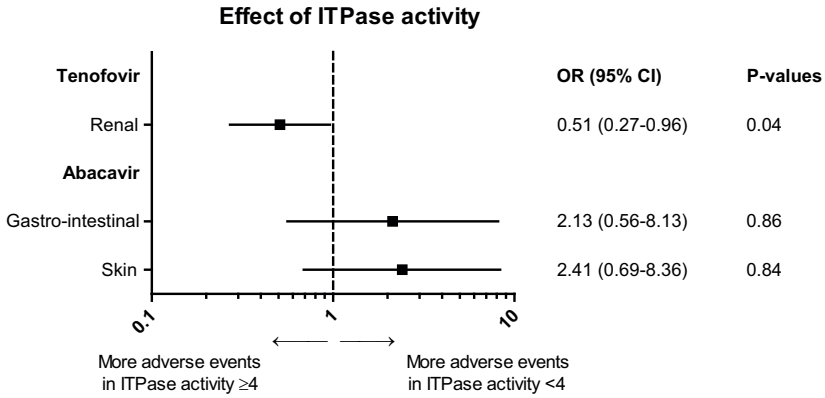

Figure 2. Effect ITPase activity on regimen associated adverse events. The effect of decreased ITPase activity on the occurrence of regimen associated adverse events (renal adverse events for tenofovir and gastro-intestinal and skin related adverse events for abacavir) is plotted. Odds ratio with 95% confidential interval and matching p-values are displayed. The analyses were conducted with repeated statement, adjusted for cumulative total duration of cART, cumulative duration of purine analogue therapy of all prior regimens and duration of the current regimen.

Table 2. Occurrence of adverse events in patients grouped by ITPase activity, percentage of adverse events and crude p-values per purine type.

cART containing:	ITPase activity <4[a]		ITPase activity ≥4[a]		Crude p-value
	AE[b], n / Patients, n	% of total AE[b]	AE[b], n / Patients, n	% of total AE[b]	
Tenofovir	137/306	45.1%	167/295	54.9%	0.004
Abacavir	74/131	61.2%	47/113	38.8%	0.02
Didanosine	27/51	40.3%	40/77	59.7%	0.91
No purine	118/246	53.2%	104/203	46.8%	0.49

[a] mmol IMP/mmol Hb/hour; [b] AE, adverse events

Didanosine and regimens without tenofovir, abacavir and didanosine

No statistically significant association was found for AEs occurring with use of didanosine or regimens without tenofovir, abacavir and didanosine and ITPase activity.

Association of *ITPA* genotype with AEs

The occurrence of AEs and the effect of the *ITPA* genotype are displayed in Table 3 and Figure 3 respectively. 960 regimens were prescribed for patients with wt/wt genotype and 435 for patients with one or more of the SNPs. Genotype was unknown for 27 regimens.

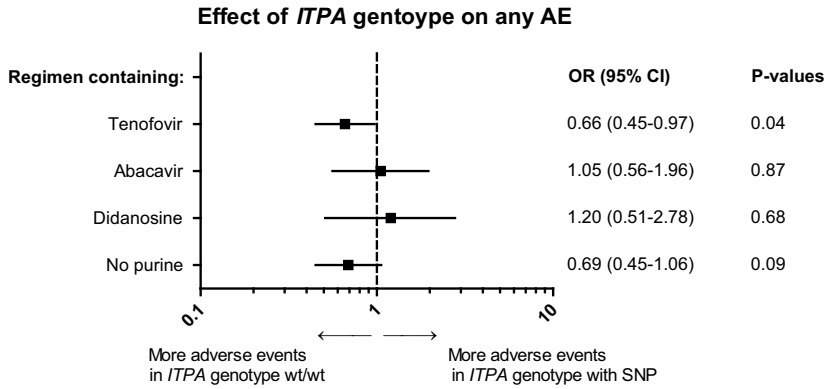

Figure 3. Effect *ITPA* genotype on total adverse events. The effect of *ITPA* genotype wt/wt versus the other *ITPA* genotypes on the occurrence of total adverse events (grouped by regimens containing tenofovir, abacavir or didanosine and regimens without tenofovir, abacavir or didanosine) is plotted. Other *ITPA* genotypes than wt/wt were wt/c.124+21A>C, wt/c.94C>A, c.124+21A>C/c.124+21A>C, c.94C>A/c.94C>A or c.124+21a>C/c.94C>C. Odds ratio with 95% confidential interval and matching p-values are displayed. The analyses were conducted with repeated statement, adjusted for cumulative total duration of cART, cumulative duration of purine analogue therapy of all prior regimens and duration of the current regimen.

Tenofovir

ITPA genotypes other than wt/wt, associated with decreased ITPase activity, seemed to be protective against AEs (p=0.04), as 72.4% of all AEs occurred in the patients with *ITPA*

Table 3. Occurrence of adverse events in patients grouped by *ITPA* genotype, and crude p-values per purine type for each type of adverse event.

cART containing:	SNP in *ITPA* genotype[a]		*ITPA* genotype wt/wt		*ITPA* genotype unknown		Crude p-value
	AE[b], n / Patients, n	% of total AE	AE[b], n / Patients, n	% of total AE[b]	AE[b], n / Patients, n	% of total AE[b]	
Tenofovir	80/186	26.3%	220/409	72.4%	4/6	1.3%	0.04
Abacavir	38/75	31.4%	82/164	67.8%	1/5	0.8%	0.52
Didanosine	18/31	26.9%	48/96	71.6%	1/1	1.5%	0.54
No purine	64/143	28.8%	151/291	68.0%	7/15	3.2%	0.36

[a] heterozygous wt/c.124+21A>C or wt/c.94C>A, homozygous c.124+21A>C or c.94C>A or compound heterozygous c.124+21A>C/c.94C>A; [b] AE, adverse events

genotype wt/wt and 26.3% in patients with SNPs in the genotype. 1.3% Of the regimens with an AE had an unknown genotype. 76.4% of all renal AEs occurred in the regimens with wt/wt genotype, this difference was not statistically significant, see Figure 4.

Abacavir

No association was found between *ITPA* genotype and all AEs in patients using abacavir. For gastro-intestinal AEs a SNP in the *ITPA* genotype was associated with an increased number of AEs (OR 3.45 for *ITPA* genotypes with SNPs versus wt/wt genotype; 95% CI 0.99–11.11; p=0.05), see Figure 4.

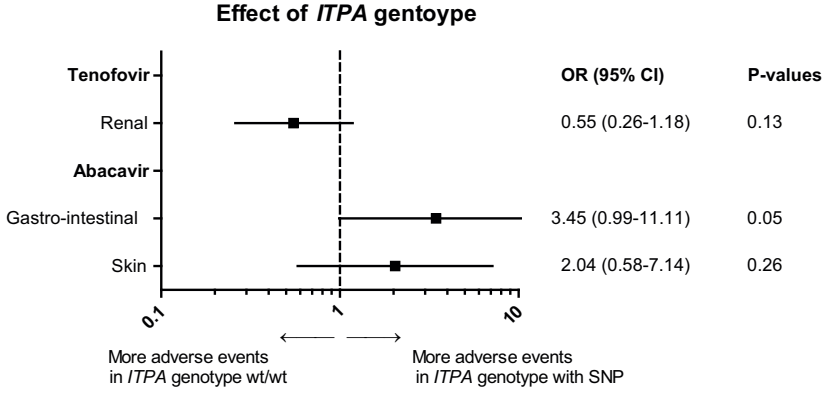

Figure 4. Effect *ITPA* genotype on regimen associated adverse events. The effect of *ITPA* genotype wt/wt versus the other *ITPA* genotypes on the occurrence of regimen associated adverse events (renal adverse events for tenofovir and gastro-intestinal and skin related adverse events for abacavir) is plotted. Other *ITPA* genotypes than wt/wt were wt/c.124+21A>C, wt/c.94C>A, c.124+21A>C/c.124+21A>C, c.94C>A/c.94C>A or c.124+21a>C/c.94C>C. Odds ratio with 95% confidential interval and matching p-values are displayed. The analyses were conducted with repeated statement, adjusted for cumulative total duration of cART, cumulative duration of purine analogue therapy of all prior regimens and duration of the current regimen.

Didanosine and regimens without tenofovir, abacavir and didanosine
No association was found between *ITPA* genotype and AEs in patients using didanosine or regimens without tenofovir, abacavir and didanosine.

Carbovir-triphosphate and tenofovir-diphosphate are no direct substrates for ITPase

To test the hypothesis that carbovir-triphosphate could be a substrate for ITPase, resulting in higher carbovir levels when ITPase activity is decreased, and more pronounced adverse effects, carbovir-triphosphate was directly used as a substrate for ITPase. Carbovir-triphosphate was not a direct substrate for ITPase and neither was tenofovir-diphosphate.

DISCUSSION

Here, we show for the first time that ITPase activity is associated with the occurrence of AEs in patients using cART containing the purine analogues tenofovir and abacavir. A significant reduction in all AEs was found in patients with decreased ITPase activity using tenofovir. Moreover, mean regimen duration was significantly longer indicating a better tolerance of tenofovir containing cART regimens in patients with decreased ITPase activity. On the other hand, patients with decreased ITPase activity using abacavir were more at risk for developing AEs. Mean regimen duration was longer in the patients with normal ITPase activity, indicating a better tolerance of abacavir in these patients. No clear association between AEs and regimens without tenofovir, abacavir or didanosine could be demonstrated.

These data suggest that ITPase activity may be used as a pharmacogenetic biomarker in patients starting cART containing tenofovir or abacavir. Up to now no other biomarkers are in use, apart from HLA-B*57:01 status to predict abacavir hypersensitivity syndrome. As tenofovir is used as a part of the regimen in the PrEP studies to prevent HIV transmission, we expect an increase in the use of tenofovir by indivuduals not infected with HIV. A test to identify individuals with increased risk of developing long term adverse effects due to tenofovir would be an extremely welcome asset. The present study suggest ITPase activity is a potential candidate. In other diseases, results of studies using *ITPA* polymorphisms to predict AEs varied with the purine analogue used. The ITPase lowering *ITPA* polymorphisms were shown to be protective against ribavirin-induced anemia in hepatitis C on treatment.[19,20] In patients with inflammatory bowel disease using azathioprine, however, adverse events occurred more frequently in patients with ITPase lowering *ITPA* polymorphisms.[7,21] In our study a lower ITPase activity was associated with less cART regimen switches due to AEs in patients using tenofovir, whereas regimens containing abacavir were more frequently switched. The underlying cause of the observed differences between the purine analogues

tenofovir and abacavir is yet unclear. Both abacavir and tenofovir are potent inhibitors of HIV reverse transcriptase.[22-24] However, the chemical structure differs greatly between the two analogues (Figure 5). Whilst tenofovir is an acyclic adenine nucleotide analogue, abacavir is the prodrug of carbovir, which is formed by removal of the cyclopropylammonia moiety attached to the purine base and is a guanosine analogue. We expect that part of the explanation is to be found in the chemistry of these compounds.

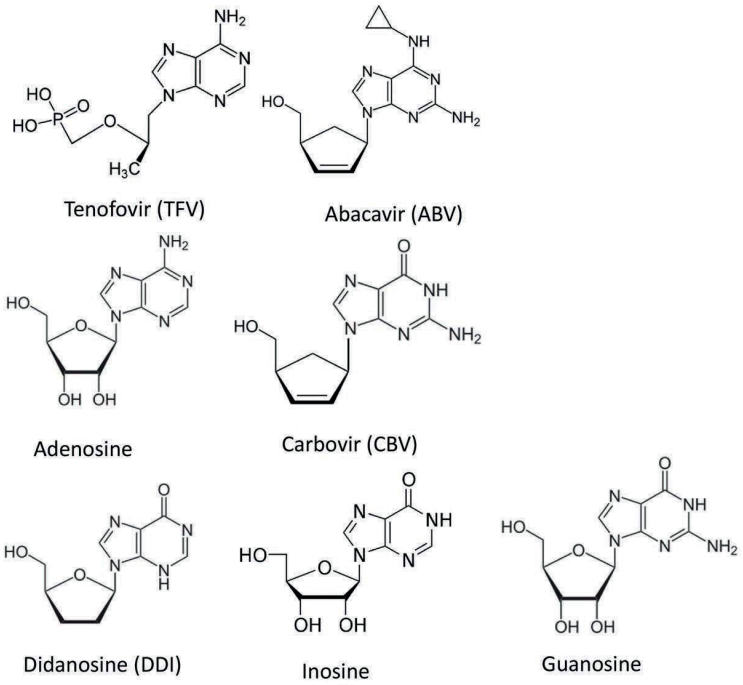

Figure 5. Chemical structures. Chemical structures of tenofovir, abacavir, adenosine, carbovir, didanosine, inosine and guanosine.

In our hands, both carbovir-triphosphate and tenofovir-diphosphate proved to be non-substrates for ITPase, so the mechanism behind our observation that decreased ITPase activity leads to an increased occurrence of adverse events in abacavir therapy is not easily explained. As was hypothesized by Coelho et al,[25] the use of cART may lead to an increase in IMP and so to an increase in cytotoxicity by an increased ITP level in patients with decreased ITPase activity. Interestingly, we observed cultured skin fibroblasts of patients with encephalopathy associated with *ITPA* mutations[9] leading to severely reduced ITPase activity show increased levels of IMP rather than ITP when compared to controls, so direct toxicity by ITP can only explain part of the mechanism involved. Bondoc and coworkers[26] demonstrated that intra-cellular anabolism of carbovir was stimulated by adenine, adenos-

ine, hypoxanthine, inosine and even dideoxy-Inosine. Their hypothesis is that increased intracellular levels of IMP and ATP enhanced the anabolism of carbovir by stimulating phosphorylation by 5'-nucleotidase. In line with this, we might hypothesize that a decreased ITPase activity leads to an increase in IMP and so to an increase of carbovir anabolism. In addition to its anti-retroviral activity, carbovir-triphosphate has been demonstrated to be a competitive inhibitor of soluble guanylate cyclase influencing platelet activity.[27] Considering that carbovir-triphosphate might be increased by decreased ITPase activity, the interference of carbovir-triphosphate in the nitric oxide (NO) signalling pathway may be considerable, leading to adverse events and regimen change. This hypothesis needs further investigation to clarify the mechanism.

Skin reactions related to abacavir are an immunological phenomenon. In our study, most of the skin related adverse events occurred in the abacavir-containing regimens used by patients with decreased ITPase actvitiy, although this difference was not statistically significant. The role of ITPase in immunologically induced AEs remains to be elucidated.

For tenofovir an explanation for the present results seems to be less straightforward. Tenofovir differs from carbovir in two essential aspects: tenofovir is a nucleoside-monophosphate and a adenosine analogue rather than a guanosine analogue. Tenofovir metabolites appear to be resistant to deamination[28] making accumulation of the deaminated tenofovir-diphosphate metabolite due to decreased ITPase activity unlikely. At this moment we can only speculate that ITPase activity influences tenofovir metabolism in a yet unknown fashion. In this study we were not able to rule out other factors that may have influenced renal events other than tenofovir use, like hypertension, diabetes mellitus, age and the use of other medication, like non-steroidal anti-inflammatory drugs.

All previous studies evaluating the effect of *ITPA* genotype polymorphisms on adverse events assumed that the reported ITPase activity directly corresponds to a specific *ITPA* polymorphism.[14,18] However, in accordance with our previous work[29] we found that an *ITPA* variant such as wt/c.124+21A>C leads to a variety of ITPase activities from as low as 1.53 to as high as 7.70 mmol IMP/mmol Hb/hour. Moreover, in spite of wt/wt genotype, HIV-infected patients were found to have a decreased ITPase activity compared to control patients[16] and therefore more often will have an ITPase activity <4 mmol IMP/mmol Hb/hour. In HIV, the association between *ITPA* genotype and ITPase activity is less strict as has previously been assumed. This may be an explanation why *ITPA* genotype correlated less to AEs compared to ITPase activity, as we found in this study. Previously we showed that ITPase activity is lower in HIV-infected patients compared to control populations in erythrocytes[16] as well as in leukocytes[30] independent of *ITPA* genotype, which did not appear to be an effect of nucleoside analogues.[16]

Some limitations of this research need to be mentioned. Because cART is, by definition, a combination of antiretroviral drugs, adverse events during a cART regimen might be attributed to more than one drug. However, by using repeated statement and adjusting the statistical analysis for cumulative total duration of cART, cumulative duration of purine analogue therapy of all prior regimens and duration of the current regimen, we were able to measure the association between ITPase activity and tenofovir and abacavir containing regimens. More studies will be needed to confirm our findings.

The lack of more significant results analyzing specific adverse events other than gastrointestinal, renal and skin related adverse events in this study may be due to the relatively small numbers of the occurrence of these adverse events. This was probably due to the fact that in our definition of adverse event we only used the reasons for stopping a regimen in combination with retraceable laboratory values. We have chosen this strategy because in the SHM database reason for switching cART regimen is a mandatory question, whereas reporting AEs is up to the physician and not a prerequisite item in the database and therefore is a less reliable parameter. Still, in 7% of the regimens, the reason for switching cART was unknown. The number of regimens with unknown reason for switching were equally distributed between both ITPase activity groups. We therefore do not expect this to be affecting the results of the study.

Because the data were collected retrospectively, the causality between the use of cART and the occurrence of AEs cannot be proven in this study. By using only the reasons for stopping the cART regimen or retraceable laboratory values, we avoided some bias, however, prospective trials are needed to confirm our data.

In conclusion, ITPase enzyme activity <4 mmol IMP/mmol Hb/hour seems to be protective against occurrence of adverse events in cART regimens containing tenofovir, while it leads to an increase in adverse events in cART regimens containing abacavir. These results warrant further elucidation of the mechanism involved and need to be confirmed in a prospective trial.

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Chapter 5.

Inosine 5'-triphosphatase activity is associated with TDF-associated nephrotoxicity in HIV.

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Submitted

ABSTRACT

Objectives

Nucleotide reverse transcriptase inhibitors play a pivotal role in HIV-treatment. The enzyme Inosine 5'-triphosphatase (ITPase) is involved in the nucleotide metabolism and has been associated with adverse drug events. We studied the association between ITPase-activity and tenofovir disoproxil fumarate (TDF)-associated nephrotoxicity.

Design

Single center 1:2 case control cohort study, including suppressed HIV-infected patients with (cases) and without (controls) TDF-associated nephrotoxicity.

Methods

26 cases (eGFR-decline >25% and/or ≥2 proximal tubular dysfunction (PTD)-markers during TDF use) were matched to 55 controls. ITPase-activity and *ITPA* genotype were measured in all patients. The primary endpoint was the proportion of patients with normal ITPase-activity (≥4 mmol IMP/mml Hb/hour) in cases versus controls. The eGFR-improvement 48 weeks after TDF-cessation was measured in cases. McNemar's test, conditional logistic regression, and paired T-tests were used.

Results

The eGFR in cases and controls at TDF-discontinuation was 78 and 85 ml/min. 19/26 cases (73.1%) versus 28/55 controls (50.9%) had normal ITPase activity, p=0.001 (OR 2.55, 95% CI 0.89-7.31, p=0.08). 23/26 cases (88.5%) versus 40/55 controls (72.7%) had wt/wt *ITPA* genotype, p=0.26 (OR 2.59, 95%CI 0.70-9.54, p=0.15). After TDF-cessation, the eGFR increased in cases with normal ITPase activity (-5.5 to +4.4 ml/min/year, p=0.008), but remained stable in cases with reduced activity (-4.3 to -4.0, p=0.97). In cases with wt/wt *ITPA* genotype, eGFR increased from –5.0 to +3.0 ml/min/year, p=0.021. 13/16 cases with PTD had normal ITPase activity. Of cases with available data, 50% with normal activity had PTD-recovery after TDF-cessation.

Conclusions

Normal ITPase-activity is associated with nephrotoxicity during TDF use and recovery after TDF-cessation. ITPase-activity might function as a screening-tool for probable occurrence and reversibility of TDF-toxicity.

INTRODUCTION

Tenofovir disoproxil-fumarate (TDF) is a recommended nucleotide-analog reverse-transcriptase inhibitor (NRTI) in combination antiretroviral therapy (cART) for HIV-treatment. Other indications for TDF-use are chronic hepatitis B virus infection and pre-exposure prophylaxis (PrEP).[1] Use of TDF is associated with an accelerated estimated glomerular filtration rate (eGFR)-decline[2-4] and proximal tubular dysfunction (PTD).[2,5,6] In clinical trials, tenofovir alafenamide (TAF), a novel tenofovir prodrug, showed comparable virological efficacy as TDF, but caused smaller eGFR-declines and renal tubular proteinuria.[7,8] Therefore, TAF-containing cART became a recommended first-line regimen next to TDF-containing cART.[9,10] Recently, generic TDF has become available, which might favor prescribing TDF over TAF for cost-effectiveness and aid in the roll out of cART in resource-limited countries. Additionally, the use of TDF as PrEP is increasing. Therefore, it is useful to predict in which patient the risk of TDF-associated nephrotoxicity is high, and whether it would recover.

DNA consists of the canonical nucleobases adenine, cytosine, guanine, and thymidine. However, incorporation of non-canonical nucleoside triphosphates in the DNA potentially causes cyto- or genotoxicity.[11] The housekeeping enzyme Inosine 5'-triphosphatase (ITPase), encoded by the polymorphic gene *ITPA* (OMIM #147520), eliminates the nucleotide pool from non-canonical nucleoside triphosphates.[12] In HIV-infected patients, ITPase activity and enzyme expression were decreased compared to non HIV-infected controls in erythrocytes and CD4+ lymphocytes,[13,14] which could not be fully explained by the single nucleotide polymorphisms (SNPs) c.94C>A (p.Pro32Thr, NCBI rs1127354) and c.124+21A>C (NCBI rs7270101) in the *ITPA* gene. In a retrospective cross-sectional study, a normal ITPase activity was associated with broadly defined nephrotoxicity in HIV-infected patients on TDF-containing cART.[15]

We evaluated whether ITPase activity or *ITPA* genotype could be useful biomarkers to predict TDF-associated nephrotoxicity, and whether they were associated with eGFR-improvement after TDF-cessation.

METHODS

This was a 1:2 matched case-control study in a cohort of HIV-1 infected adult patients from the Erasmus Medical Center, Rotterdam, the Netherlands. The study was approved by the local ethics committee, conducted according to the Helsinki Declaration, and participants provided informed consent. Participants were selected from 2 previous studies: a cohort study on TDF-associated nephrotoxicity and a randomized clinical trial in which TDF-

containing cART was discontinued (DOMONO,NCT02401828).[16,17] Cases were patients who developed TDF-associated nephrotoxicity, and controls were patients who did not. Matching was performed for gender, age and ethnicity. Nephrotoxicity was defined as >25% eGFR-decrease during TDF-use and/or presence of ≥2 PTD markers: normoglycaemic glucosuria, hypophosphatemia <0.8 mmol/L, urine protein:creatinine ratio (UPCR) >15.0 mg/mmol, urine albumin:protein ratio (APR) <0.4 in patients with increased UPCR, or increased fractional excretion of phosphate (>20%, or >10% in hypophosphatemic patients).[18] ITPase activity was measured as described previously.[19] ITPase activity ≥4 mmol IMP/mml Hb/hour was considered normal.[20] *ITPA* genotype was determined by genotyping whole blood for the *ITPA* SNPs c.94C>A (p.Pro32Thr, rs1127354) and c.124+21A>C (rs7270101). *ITPA* genotypes without these SNPs were considered wt/wt. 47 patients (15 cases and 32 controls) were selected from the study of Rokx et al, and 34 patients (11 cases and 23 controls) from the DOMONO-study.[16,17] Data on demographics, medical history (diabetes mellitus, hypertension, hepatitis C virus infection, cardiovascular disease), nephrotoxic medications (sulphamethoxazole/trimethoprim, non-steroidal anti-inflammatory drugs, angiotensin converting enzyme-inhibitors, angiotensin-2 receptor-antagonists, and valacyclovir or ganciclovir), eGFR, and PTD markers during TDF-use were collected, as well as eGFR and PTD-markers 48 weeks after TDF-cessation.

The primary outcome of this study was the proportion of normal versus reduced ITPase activity in cases versus controls. Secondary outcomes were: 1) proportions of patients with wt/wt versus wt/94C>A or wt/124+21A>C *ITPA*-genotype in cases versus controls, and 2) improvement of eGFR and PTD 48 weeks after TDF-cessation in cases with normal versus decreased ITPase activity and wt/wt versus another *ITPA* genotype. A sample size of 87 (29 cases and 58 controls) was needed to prove with a 1:2 case-control study-design that a significantly greater proportion of patients with nephrotoxicity had normal ITPase activity (π_1=0.5) than patients without nephrotoxicity (π_2=0.2), based on previous findings, with power 1-β=80% and a 2-sided α of 0.05.[15] McNemar's Test was used to compare proportions of patients with normal ITPase activity in cases and controls. Nephrotoxicity related to both ITPase activity (normal versus reduced) and *ITPA* genotype (wt/wt versus genotype with SNP) was analyzed using conditional logistic regression, resulting in an odds ratio (OR) with 95% confidence interval (CI). Fishers' Exact Test, Unpaired T-tests, Chi Square Tests, and Mann Whitney U Tests were used for other comparisons between patients with normal versus reduced ITPase-activity, and paired T-tests were performed for comparisons on eGFR-improvement. An alfa of 0.05 was used.

RESULTS

81 Patients were included, of whom 26 patients were cases and 55 patients were controls. Although we intended to include 87 patients based on our sample size calculation, we did a preliminary analysis after including 81 patients due to repeated non-adherence to scheduled outpatient appointments of the remaining eligible patients. This analysis showed highly significant results for the primary endpoint, with a calculated power of 76%. Therefore, patient inclusion was stopped for ethical arguments, since we considered that the supporting data for our assumptions for the power calculation were limited and could deviate from the true difference. In both groups, participants were predominantly Caucasian middle-aged males. The duration of TDF-use was comparable between cases and controls (83 and 84 months), as well as use of nephrotoxic co-medication and comorbidity. The mean (SD) eGFR in cases and controls at the moment of TDF-discontinuation was 78 (19) and 85 (13) ml/min. Of the cases, 80.8% had >25% eGFR-decline since TDF initiation, 61.5% had ≥2 PTD-markers, and 42.3% had both. (Table 1).

Of the cases, 73.1% (19/26) had a normal ITPase activity versus 50.9% (28/55) of controls (p=0.001; Table 2). Wt/wt *ITPA* genotype was present in 88.5% of cases and 72.7% (40/55) of controls (p=0.26; Table 2). Conditional logistic regression showed an increased and nearly statistically significant risk for nephrotoxicity in patients with normal ITPase activity and *ITPA* genotype wt/wt: OR 2.56 (95%CI 0.89-7.31; p=0.08), and OR 2.59 (95%CI 0.70-9.54; p=0.15), respectively. The eGFR-course improved from -5.5 ml/min/year during TDF to +4.4 ml/min/year after TDF-cessation (p=0.008) in cases with normal ITPase activity (N=19), whereas it remained stable in cases with reduced activity (N=4): -4.3 versus -4.0 ml/min/year, p=0.97. (Table 2). Of 11 cases that started dolutegravir therapy after TDF-cessation, 7 had normal ITPase activity, of whom 6 had improvement of eGFR, whereas 4 had reduced activity, of whom 1 had improvement of eGFR. These results indicate that patients with a normal ITPase activity may recover from TDF-associated nephrotoxicity after TDF-cessation, but patients with reduced activity may not. In cases with wt/wt *ITPA* genotype (N=22), the eGFR-course improved from -5.0 to +3.0 ml/min/year, p=0.021 (Table 2). eGFR data from only 1 patient with SNPs in the *ITPA* genotype were available, of which no conclusions can be drawn. Of the 16 cases with PTD, 3 had decreased and 13 had normal ITPase activity. Week 48 data were available in 9 patients, of whom the only patient with decreased ITPase activity had no PTD recovery, and PTD recovered in 4 of 8 patients with normal activity.

Table 1. Clinical characteristics of the patients with (cases) and without (controls) TDF-associated nephrotoxicity.

Characteristic	**Cases (n=26)**	**Controls (n=55)**	**p-value**
Male gender, n (%)	23 (88.5)	49 (89.1)	1.00 (FE)[a]
Age, mean years ± SD[b]	51 ± 10	52 ± 9	0.50 (UT)[c]
Ethnicity, n (%)			0.87 (FE)[a]
Caucasian	22 (84.6)	44 (80.0)	
African	2 (7.7)	3 (5.5)	
Asian	0 (0.0)	1 (1.8)	
Latino	2 (7.7)	7 (12.7)	
Route of transmission, n (%)			0.77 (FE)[a]
MSM[d]	20 (76.9)	37 (67.3)	
Heterosexual	5 (19.2)	12 (21.8)	
IVDU[e]	1 (3.8)	3 (5.5)	
Unknown	0 (0.0)	3 (5.5)	
Smoking status, n (%)			0.60 (CS)[f]
Current	8 (30.8)	22 (40.0)	
Previous	8 (30.8)	12 (21.8)	
Never	9 (34.6)	21 (38.2)	
Unknown	1 (3.8)	0 (0.0)	
Comorbidities, n (%)			
Hypertension	5 (19.2)	10 (18.2)	1.00 (FE)[a]
Diabetes mellitus	3 (11.5)	1 (1.8)	0.10 (FE)[a]
Hepatitis C virus infection	2 (7.7)	5 (9.1)	1.00 (FE)[a]
Cardiovascular disease	3 (11.5)	4 (7.3)	0.68 (FE)[a]
TDF[g]-containing cART[h], n (%)			0.86 (FE)[a]
+ NNRTI[i]-containing, N (%)	24 (92.3)	49 (89.1)	
RPV[j]-containing	9 (34.6)	18 (32.7)	
+ bPI[k]-containing, N (%)	2 (7.7)	5 (9.1)	
+ INSTI[l]-containing, N (%)	0 (0.0)	1 (1.8)	
Median duration of TDF-use[m], months (Q1,Q3)	83 (50,117)	84 (46,115)	0.72 (MWU)[n]
Mean eGFR[o] at discontinuation of TDF[g], ml/min, ± SD[b]	78.1 ± 19.2	85.2 ± 12.9	0.09 (UT)[c]
Comedication during TDF[g]-use, n (%)			
Sulfamethoxazol/trimethoprim	0 (0.0)	1 (1.8)	1.00 (FE)[a]
ACE[p]-inhibitor	3 (11.5)	3 (5.5)	0.38 (FE)[a]
AT2[q]-antagonist	0 (0.0)	4 (7.3)	0.30 (FE)[a]
Acyclovir/gancyclovir	1 (3.8)	3 (5.5)	1.00 (FE)[a]
NSAID[r]	7 (26.9)	18 (32.7)	0.62 (CS)[f]

Table 1. Clinical characteristics of the patients with (cases) and without (controls) TDF-associated nephrotoxicity. (continued)

Characteristic	Cases (n=26)	Controls (n=55)	p-value
TDF[g]-associated nephrotoxicity, n (%)			
>25% decrease in eGFR[o]	21 (80.8)		
≥2 markers of PTD[s]	16 (61.5)		
>25% decrease in eGFR[o] + ≥2 markers of PTD[s]	11 (42.3)		

[a] FE, Fisher's Exact test; [b] SD, standard deviation; [c] UT, Unpaired T-test; [d] MSM, men who have sex with men; [e] IVDU, intravenous drug use; [f] CS, Chi Square test; [g] TDF, tenofovir disoproxil fumarate; [h] cART, combination antiretroviral therapy; [i] NNRTI, non-nucleoside reverse transcriptase inhibitor; [j] RPV, rilpivirine; [k] bPI, boosted protease inhibitor; [l] INSTI, integrase strand transfer inhibitor; [m] duration of TDF-use at inclusion and allocation to 'case' or 'control'; [n] MWU, Mann Withney U test; [o] eGFR, estimated glomerular filtration rate; [p] ACE, angiotensin converting enzyme; [q] AT2, Angiotensine 2 antagonist; [r] NSAID, Non-steroidal anti-inflammatory drug; [s] PTD, proximal tubular dysfunction.

Table 2. Renal outcomes related to ITPase-activity and *ITPA* genotype.

	TDF[a]-associated nephrotoxicity		P-value	eGFR[b]-change[c] in cases, mean ±SD[d]		P-value
	Yes (n=26)	No (n=55)		during TDF[a]-use	after TDF[a] discontinuation	
ITPase activity[e]			0.001 (MN[f])			
<4	7 (26.9%)	27 (49.1%)		-4.3 ± 5.8	-4.0 ± 9.9	0.97 (PT[g])
≥4	19 (73.1%)	28 (50.9%)		-5.5 ± 4.8	+4.4 ±13.0	0.008 (PT[g])
Mean ± SD[d]	4.41 ± 1.28	3.90 ± 1.31	0.103 (UT[h])			
***ITPA* genotype**			0.256 (MN[f])			
Wt/94C>A	2 (7.7%)	3 (5.5%)		*	*	*
Wt/124+21A>C	1 (3.8%)	12 (21.8%)		*	*	*
Wt/wt	23 (88.5%)	40 (72.7%)		-5.0 (4.7)	+3.0 (13.0)	0.021 (PT[g])

[a] TDF, tenofovir disoproxil fumarate; [b] eGFR, estimated glomerular filtration rate; [c] in ml/min/year, [d] SD, standard deviation; [e] mmol IMP/mmol Hb/hour; [f] MN, McNemar (Wt/wt versus wt/94C>A and wt/124+21A>C combined); [g] PT, Paired samples T-test; [h] UT, Unpaired T-test; * indicates that not enough data were available for analysis.

DISCUSSION

In this case-control and cohort study in HIV-patients, TDF-associated nephrotoxicity was associated with a normal ITPase activity, and in these patients their eGFR-course ameliorated after TDF-cessation. Less patients with reduced ITPase activity had and accelerated eGFR-decline, which did not recover after TDF-cessation. ITPase activity may be used as biomarker to predict which patients are at high risk for developing nephrotoxicity during TDF-use (more pronounced in normal ITPase activity), in which patients TDF-associated nephrotoxicity may be irreversible (decreased activity), and in whom TDF therefore should

be discontinued when signs of TDF-associated nephrotoxicity occur. The results of the present study confirm our previous findings that a normal ITPase activity was associated with nephrotoxicity during TDF-use.[15] However, in this study, we were able to strictly define nephrotoxicity and investigate the association between ITPase activity and TDF-associated nephrotoxicity.[16]

It is unclear why a normal ITPase activity is associated with both TDF-associated nephrotoxicity and recovery after TDF-cessation. TDF causes mitochondrial DNA (mtDNA) toxicity in tubular cells.[21-23] Imbalanced mitochondrial nucleotide pools can cause mtDNA depletion, resulting in mitochondrial dysfunction.[24,25] Furthermore, TDF leads to increased oxidative stress in mitochondria of renal tubular cells.[26] In cells with oxidative stress, the enzyme xanthine oxidase (XO) activity is relatively increased[27] and XO is a source of free radicals[28]. A normal ITPase activity, compared to decreased activity, may lead to more availability of hypoxanthine (formed from inosine), a substrate for XO. Eventually, the combination of a normal ITPase activity and TDF-use may lead to increasing oxidative stress, resulting in nephrotoxicity. Further research is warranted to clarify whether erythrocyte ITPase activity is an adequate surrogate for ITPase activity in renal cells, and what the effect of ITPase on the nucleotide pools in renal mitochondria is. Differences in ITPase activity in mitochondria, the effect of the ITPase activity on mitochondrial TDF-metabolism, and the role of TDF in oxidative stress should be studied.

This study has some limitations. First, the sample size of the study was based on previous findings,[15] but data on ITPase activity related to TDF-associated nephrotoxicity are scarce and difficult to translate to assumptions for our sample size. As the results of a preliminary analysis in the first 81 included patients were already highly significant, the final 6 patients were not included. Second, we cannot exclude that the nephrotoxicity observed in our cases was due to other, unidentified, factors, although patient characteristics were comparable between the cases and controls. Besides, data on longer follow-up were not available, and therefore we cannot exclude that patients who are included as controls, could have developed nephrotoxicity with longer use of TDF. Third, recovery of nephrotoxicity may be underestimated in patients using DTG. DTG is known for its inhibitory effect on tubular creatinine clearance, leading to an increase in serum creatinine, which decreases the eGFR without impairment of actual glomerular or tubular function.[29] 11 of the 26 cases were former DOMONO-participants, and switched to DTG monotherapy. Indeed, in some of our patients using DTG the eGFR further decreased, but this was in only 1 of 7 patients with normal ITPase activity, versus in 2/4 cases with reduced activity. So even after a switch from TDF to DTG, the distinct between normal versus reduced ITPase activity in relation to eGFR-improvement remains. Given the low numbers of patients with follow-up of PTD-

markers after 48 weeks, importantly due to the observational nature of the study of Rokx et al, we were not able to provide data on recovery of PTD.

In conclusion, ITPase activity is associated with nephrotoxicity during TDF-use for HIV-infection and could be used to predict eGFR-recovery, but the underlying mechanism needs to be elucidated. ITPase activity may be used in the decision to initiate and discontinue TDF in an individual patient, and this recommendation should be confirmed in a prospective trial.

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Chapter 6.

Metabolic events in HIV-infected patients using abacavir are associated with erythrocyte inosine triphosphatase activity.

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ABSTRACT

Objectives

Abacavir use has been associated with an increased risk of cardiovascular disease (CVD) and metabolic events in HIV-infected patients, although this finding was not consistently found. It is unclear whether abacavir only increases this risk in subpopulations of HIV-infected patients. It may be hypothesized that inosine 5'-triphosphate pyrophosphohydrolase (ITPase), an enzyme involved in the metabolism of purine analogues used in HIV treatment, plays a role in the risk of CVD and metabolic events in HIV-infected patients.

Methods

ITPase activity and *ITPA* genotype were determined in 393 HIV-infected patients. ITPase activity <4 mmol IMP/mmol Hb/h was considered decreased. *ITPA* polymorphisms tested were: c.94C>A (rs1127354) and c.124+21A>C (rs7270101). ORs were determined using generalized estimating equation models for developing CVD in patients who had ever been exposed to abacavir, tenofovir or didanosine and for developing metabolic events in patients currently using these drugs.

Results

In patients using abacavir, metabolic events were associated with ITPase activity. No association was demonstrated for tenofovir or didanosine. The OR for metabolic events was 3.11 in patients using abacavir with normal ITPase activity (95% CI 1.34–7.21; p=0.008) compared with patients with decreased ITPase activity (adjusted for age, BMI, cumulative duration of combination antiretroviral therapy (cART) use and the use of PI and NNRTI). CVD was not associated with ITPase activity or *ITPA* genotype.

Conclusions

This study shows, for the first time, that ITPase activity is associated with the occurrence of metabolic events in patients using abacavir. Further studies are needed to confirm this association and to elucidate the possible mechanism.

INTRODUCTION

The life expectancy of patients infected with HIV has improved substantially in recent decades[1] and in developed countries has been approaching that of the non-HIV-infected general population.[2] In the Netherlands, currently, 42% of HIV patients are older than 50 years of age[3] and in the USA, at year-end in 2015, the group of patients aged 50–54 years made up the largest percentage of persons living with HIV (18%).[4] With increasing age, non-communicable comorbidities such as cardiovascular disease (CVD) increase, especially among HIV-infected patients using combination antiretroviral therapy (cART).[5–7] For CVD it is unclear what the contribution of the HIV infection itself is compared with the drugs that are part of the cART regimens. In a meta-analysis the pooled relative risk of CVD among HIV-infected patients without treatment was 1.61 compared with people not infected with HIV.[8] The pooled relative risk for HIV-infected patients on treatment was 2.00 compared with non-HIV-infected people.[8] Different antiretroviral medication classes have been associated with a higher risk of CVD. The relative risk of CVD was 1.41 (95% CI 1.2–1.65) for patients on PI-based cART compared with those on non-PI-based cART.[8] An increased rate of myocardial infarction in patients recently treated with abacavir and didanosine was first reported in the D:A:D study.[9] Since then, several cohort studies and randomized clinical trials have confirmed the association of abacavir use and increased risk for CVD[10–12] and in a meta-analysis the pooled relative risk was 1.8 (95% CI 1.43–2.26; $p<0.001$) for patients treated with abacavir.[8] Currently this association is still debated, as other studies have found either no effect or no significant effect of abacavir use on the risk of CVD.[13,14]

The pathogenic mechanism of the potential association between abacavir use and CVD is not clear. Which factors predispose patients using abacavir to a higher risk of CVD, apart from the classic risk factors such as dyslipidaemia, hypertension (HT) and smoking, has not yet been elucidated. It may be hypothesized that differences in the metabolism of abacavir predispose to a higher risk for CVD. The active metabolite of abacavir is carbovir triphosphate, which is a guanosine analogue. Guanosine 5'-triphosphate (GTP) is a low-affinity substrate for the enzyme inosine 5'-triphosphate pyrophosphohydrolase (ITPase) compared with the natural substrate inosine 5'-triphosphate (ITP).[15] ITPase is one of the scavenger enzymes eliminating the potentially cytotoxic or genotoxic non-canonical nucleoside triphosphates from the nucleotide pool[16] and is encoded by the *ITPA* gene on chromosome 20p (OMIM #147520). The SNPs c.94C>A (p.Pro32Thr, NCBI rs1127354) and c.124+21A>C (NCBI rs7270101) in the *ITPA* gene can cause a decrease in ITPase activity.[17] In HIV-infected patients, ITPase was found to have decreased expression in leukocytes, which was not associated with *ITPA* genotype,[18] and decreased activity in erythrocytes in

patients with *ITPA* genotype wt/wt or c.94C>A compared with a control population with the same genotype.[19]

The natural role of ITPase, the different ITPase activity in HIV-infected patients and the potential role of ITPase in the metabolism of the purine analogues used in HIV treatment, suggest that ITPase activity may play a role in the risk of CVD in patients using abacavir.

We therefore determined whether ITPase activity and *ITPA* genotype are associated with the occurrence of CVD and with risk factors such as HT, hypercholesterolaemia and diabetes mellitus (DM) during abacavir use in a cohort of HIV-infected patients.

PATIENTS AND METHODS

Patients

HIV-infected patients at the outpatient clinic of the Maastricht University Medical Center in Maastricht, The Netherlands, who were treated with cART, were included in this study after providing written informed consent. The data used for this study were collected as described previously.[20] The study was performed according to the Helsinki Declaration and approved by the Medical Ethics Committee of the Maastricht University Medical Center, Maastricht, The Netherlands.

Endpoints

The endpoints of the study were CVD and metabolic events. CVD was defined as: reported cerebrovascular accident (CVA) (transient ischaemic attack, haemorrhagic/ischaemic/unspecified CVA), arterial occlusion, peripheral stent or bypass, coronary event (coronary artery bypass graft, percutaneous intervention or myocardial infarction) or anamnestic complaints of peripheral claudication, during the use of cART. Metabolic events were defined as: dyslipidaemia as the reported reason for stopping the cART regimen, laboratory-confirmed dyslipidaemia (total cholesterol >6.5 mM, triglycerides >2.3 mM, LDL >4.5 mM), use of lipid-lowering therapy (statins and/or fibrates), DM or HT. DM was defined as fasting glucose ≥7 mM (126 mg/dL) measured on two or more consecutive occasions within 3 months, or glucose ≥11.1 mM (200 mg/dL) measured once and in combination with symptoms of hyperglycaemia, or if the patient was reported as being diabetic, or if the patient had glucose-lowering therapy.[21] HT was defined as mean systolic pressure >140 mmHg, mean diastolic pressure >90 mmHg or if the patient used anti-hypertensive medication.[22]

ITPase activity

Erythrocyte ITPase activity was determined as described previously[23] and assessed by formation of inosine 5'-monophosphate (IMP) from ITP. ITPase activity was expressed as millimoles (mmol) of IMP formed from ITP in 1 h per mmol hemoglobin (mmol IMP/mmol Hb/h). ITPase activity of ≥4 mmol IMP/mmol Hb/h was considered normal, which is the lowest value within the 95% CI for *ITPA* wild-type (wt/wt) carriers.[19,24]

ITPA genotype analysis

ITPA genotype was analysed as described previously by Bierau et al.[19] The Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) was used to isolate genomic DNA from whole blood. The DNA was genotyped using Sanger sequencing for the two *ITPA* polymorphisms; c.94C>A (p.Pro32Thr, rs1127354) and c.124+21A>C (rs7270101). The genotype was considered to be wild-type (wt/wt) when neither of the polymorphisms was detected. All sequences were evaluated by two independent laboratory experts.

Statistical analysis

All analyses were performed with IBM SPSS Statistics 21 (IBM Corporation, NY, USA) and the statistical software package R (free download from www.rproject.org) version 3.4.3. Pearson's χ^2 tests, Fisher's exact test, independent samples T-tests and Mann–Whitney U tests were used to determine significant differences.

The occurrence of CVD was analysed using generalized estimating equation models to account for repeated statement and adjusted for differences in CVD risk profile: age at the time of changing the cART regimen, the last measured BMI, smoking status (current or former smoker), pre-existing CVD before the start of cART, HT, DM, dyslipidaemia and gender. Further adjustment for cumulative total duration of cART and use of a PI or an NNRTI at the time of the event was performed. Numerical variables were standardized in order to have the same scale.

The occurrence of a metabolic event was analysed using generalized estimating equation models to account for repeated statement and adjusted for cumulative total duration of cART, age at the time of changing the cART regimen, the last BMI measured, use of a PI and use of an NNRTI at the time of the event. Numerical variables were standardized in order to have the same scale.

Since tenofovir disoproxil fumarate (further referred to as tenofovir) and didanosine are also purine analogues, their active metabolites being adenine nucleotide analogues instead of guanosine nucleotide analogues such as carbovir, the association of ITPase activity, *ITPA* genotype and CVD in patients using these drugs in their cART regimen was determined in

addition. For exposure to either abacavir, tenofovir or didanosine, in the analysis of CVD we used current (which means use at the time of the event) and past exposure. Only current exposure was used in the analysis of metabolic events.

When abacavir, tenofovir or didanosine were used concomitantly in one cART regimen, this regimen was excluded from the analysis. P-values <0.05 were considered to be statistically significant. We did not correct for multiple testing because our analyses were hypothesis driven.

RESULTS

Patient characteristics

After excluding regimens containing a combination of didanosine, tenofovir or abacavir, 1422 regimens in 393 patients were used in the analysis.[20] Of these patients, 52.2% had an ITPase activity of <4 mmol IMP/mmol Hb/h (Table 1). No statistically significant difference was found between patients with decreased ITPase activity and with normal ITPase activity with regard to age, gender, race, CD4 nadir counts and PI and NNRTI use. In 60.1% of regimens containing no purine, a PI was part of the cART regimen; this was, respectively, 34.5%, 22.9% and 60.2% in regimens containing tenofovir, abacavir or didanosine. Current exposure to the purine analogues tenofovir, abacavir and didanosine was frequent (n=601 (42.3%), n=244 (17.2%) and n=128 (9.0%) respectively). The number of regimens with current plus past exposure to a purine analogue was 699 (49.2%) for tenofovir, 465 (32.7%) for abacavir and 365 (25.7%) for didanosine. CVD was present in 12.5% of the patients (n=49) and reported to be CVA (n=15), arterial occlusion (n=2), peripheral stent or bypass (n=6), coronary arterial bypass graft (n=6), percutaneous intervention (n=12), myocardial infarction (n=7) or anamnestic complaints of peripheral claudication (n=1). In patients with current or past exposure to abacavir, 24 CVD events occurred; CVA (n=7), coronary artery bypass grafting (n=3), percutaneous coronary intervention (n=7), myocardial infarction (n=2), arterial occlusion (n=2) and peripheral stent or bypass (n=3). In 749 regimens (52.7%) a metabolic event was found. An overview of different components of the combined endpoint metabolic event is presented in Table 2.

Effect of ITPase activity and *ITPA* genotype on CVD

No significant difference in CVD between the regimens used in patients with decreased versus normal ITPase activity was found, irrespective of cART regimen used (22 versus 27 events, respectively; crude p=0.34). ITPase activity in combination with current or past exposure to abacavir was not associated with a higher number of CVD events compared with tenofovir or didanosine exposure in our cohort (Table 3).

Table 1. Demographic and clinical characteristics of the patients (n=393) with ITPase activity <4 and ≥4 mmol IMP/mmol Hb/hour.

Characteristic	ITPase activity[a]		P-value
	<4 (n=205)	≥4 (n=188)	
Age*; mean years ± SD[b]	50.1 ± 11.1	49.7 ± 11.9	0.78
Male gender,* n (%)	164 (80.0%)	155 (82.4%)	0.53
Race, n (%)*			0.81
Caucasian	164 (80.0%)	147 (78.2%)	
Hispanic	5 (2.4%)	4 (2.1%)	
African	22 (10.7%)	27 (14.4%)	
Asian or other	14 (6.9%)	10 (5.3%)	
Mean ITPase activity* ± SD	2.44 ± 1.12	5.24 ± 1.09	<0.001
ITPA* genotype, n (%)			<0.001
Wt/wt	90 (43.9%)	175 (93.1%)	
Wt/c.124+21A>C	59 (28.8%)	9 (4.8%)	
Wt/c.94C>A or other[c]	53 (25.9%)	-	
Unknown	3 (1.5%)	4 (2.1%)	
Alcohol use*, n (%)			0.51
<2 (IU/day)	157 (76.6%)	133 (70.7%)	
≥2 (IU/day)	35 (17.1%)	36 (19.1%)	
Unknown	13 (6.3%)	19 (10.1%)	
Smoking, n (%)			0.65
Never	18 (8.8%)	21 (11.2%)	
Current or former	130 (63.4%)	116 (61.7%)	
Not current, unknown if ever	55 (26.8%)	46 (24.5%)	
Unknown	2 (1.0%)	5 (2.7%)	
Body mass index, n (%)			0.09
Underweight (<18.5)	14 (6.8%)	3 (1.6%)	
Normal (≥18.5-<25)	102 (49.8%)	95 (50.5%)	
Mild/Moderate obesity (≥25-<30)	59 (28.8%)	52 (27.7%)	
Severe obesity (≥30)	10 (4.9%)	15 (8.0%)	
Unknown	20 (9.8%)	23 (12.2%)	
Median CD4 nadir*, x10^6 cells/L (range)	207 (1-1022)	209 (3-612)	0.40
Median year of start cART* (range)	2006 (1987-2013)[c]	2006 (1987-2013)[d]	0.25
Total number of cART regimens*	734	688	
Type cART, n (%)			0.09
NNRTI[d]	342 (46.6%)	295 (42.9%)	
PI[e]	239 (32.6%)	267 (38.8%)	
NNRTI[d] and PI[e]	59 (8.0%)	45 (6.5%)	
Other	94 (12.8%)	81 (11.8%)	

* previously published; [a] mmol IMP/mmol Hb/hour; [b] SD, standard deviation; [c] Other = homozygous c.124+21A>C or homozygous c.94C>A or heterozygous c.124+21A>C/c.94C>A; [d] NNRTI, nucleoside/nucleotide analog reverse-transcriptase inhibitor; [e] PI, Protease inhibitor

Table 2. Diabetes mellitus, dyslipidemia and use of blood pressure medication or cholesterol lowering therapy for regimens used in patients with decreased and normal ITPase activity.

	All regimens		**P-values**	**Regimens currently containing abacavir**		**P-values**
	ITPase activity[a]			**ITPase activity[a]**		
	<4 (n=734)	**≥4 (n=688)**		**<4 (n=131)**	**≥4 (n=113)**	
Diabetes, n (%)			0.03			0.04
Yes	28 (3.8)	45 (6.5)		3 (2.3)	11 (9.7)	
Unknown	-	18 (2.6)		-	-	
Hypertension, n (%)			0.03			0.16
Yes	146 (19.9)	171 (24.9)		26 (19.8)	31 (27.4)	
Unknown	-	-		-	-	
Dyslipaemia, n (%)			0.01			0.17
Yes	195 (26.6)	226 (32.8)		40 (30.5)	44 (38.9)	
Unknown	-	-		-	-	
Cholesterol lowering therapy, n (%)			0.014			0.68
Yes	86 (11.7)	117 (17.1)		20 (15.3)	23 (20.4)	
Unknown	10 (1.4)	9 (1.3)		1 (0.8)	1 (0.9)	

[a] mmol IMP/mmol Hb/hour

Table 3. CVD and metabolic events for regimens used in patients with decreased and normal ITPase activity and different *ITPA* genotypes.

	ITPase activity[a]		**Crude p**	**Adjusted p**	***ITPA* genotype**		**Crude p**	**Adjusted p**
	<4	**≥4**			**Other[b]**	**wt/wt**		
	n / Regimens	**n / Regimens**			**n / Regimens**	**n / Regimens**		
CVD								
Total[c] n=1422	22/734	27/688	0.34	0.82	13/435	35/960	0.72	0.86
Tenofovir[d] n=699	12/354	17/345	0.31	0.50	7/211	21/478	0.35	0.77
Abacavir[e] n=465	11/245	13/220	0.49	0.40	6/132	18/328	0.86	0.41
Didanosine[f] n=365	2/165	6/200	0.30	0.45	0/91	8/272	0.24	n.a.
Metabolic events								
Total[c] n=1422	356/734	393/688	0.001	0.12	202/435	537/960	0.001	0.14
Tenofovir[g] n=601	155/306	168/295	0.12	0.55	89/186	231/409	0.13	0.53
Abacavir[e] n=244	63/131	82/113	<0.0001	0.008	39/75	106/164	0.003	0.24
Didanosine[h] n=128	25/51	37/77	0.92	0.75	12/31	49/96	0.26	0.61

[a] mmol IMP/mmol Hb/hour; [b] heterozygous wt/c.124+21A>C or wt/c.94C>A or homozygous c.124+21A>C or homozygous c.94C>A or compound heterozygous c.124+21A>C/c.94C>A; [c] Genotype unknown in 27 regimens; [d] Genotype unknown in 10 regimens; [e] Genotype unknown in 5 regimens; [f] Genotype unknown in 2 regimens; [g] Genotype unknown in 6 regimens; [h] Genotype unknown in 1 regimen

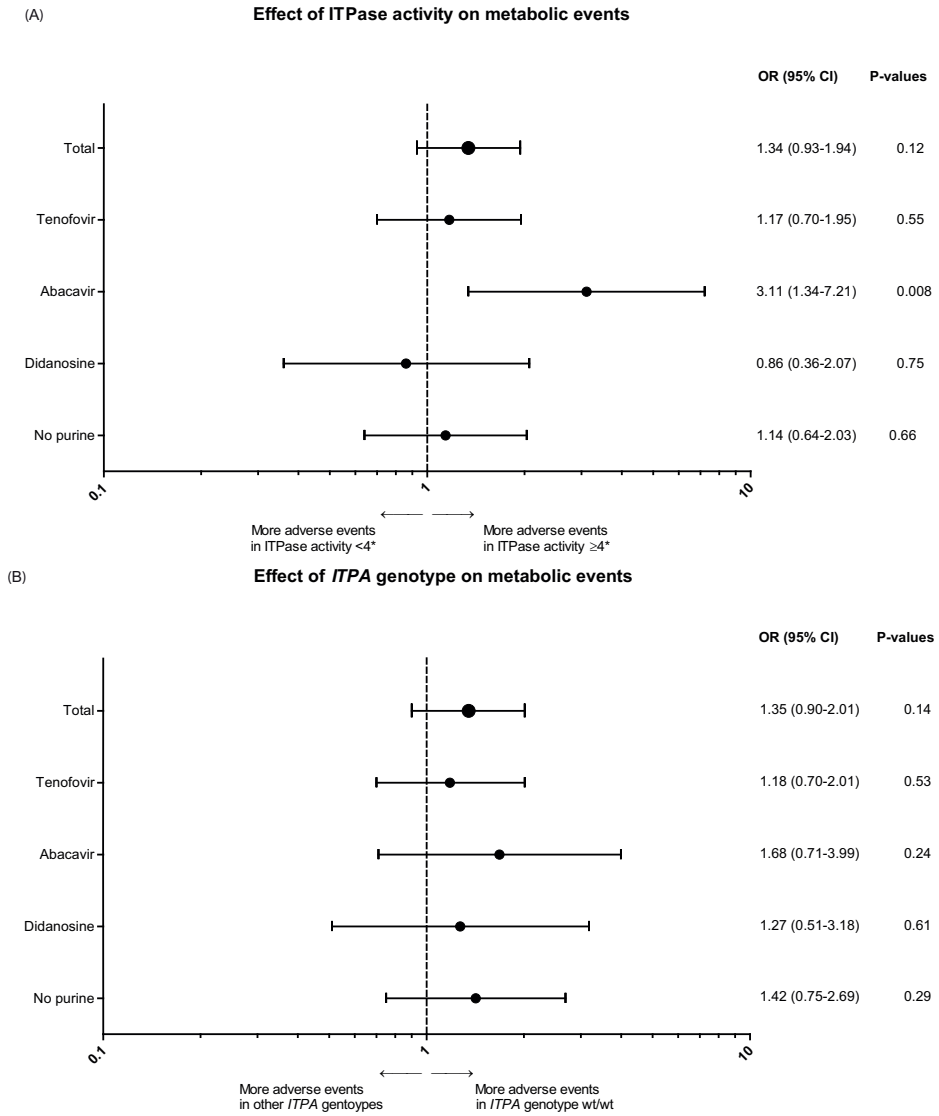

Figure 1: Effect ITPase activity (A) and *ITPA* genotype (B) on metabolic events. The effect of decreased versus normal ITPase activity (A) and of other *ITPA* genotypes versus *ITPA* genotype wt/wt (B) on the occurrence of metabolic events are plotted for all regimens (Total), and for regimens containing tenofovir, abacavir, didanosine or no purine. Odds ratio (OR) with 95% confidential interval and matching p-values are displayed. * mmol IMP/mmol Hb/hour

Regardless of cART regimen, CVD more often occurred in patients with *ITPA* genotype wt/wt versus the other *ITPA* genotypes [CVD: n=35 (71.4%) versus n=13 (26.5%) respectively; although this difference was not statistically significant (p=0.72)] (Table 3). One CVD oc-

curred in a patient with unknown *ITPA* genotype. Adjusting the data for CVD risk profiles, cumulative cART use and PI and NNRTI use did not change the outcome for CVD.

Effect of ITPase activity and *ITPA* genotype on metabolic events

Of all the metabolic events, 52.5% occurred during regimens prescribed to patients with normal ITPase activity (crude p=0.001) (Table 3). In 72.6% of the regimens currently containing abacavir used by patients with normal ITPase activity, a metabolic event occurred, compared with 48.5% in patients with decreased ITPase activity (p<0.0001; Table 3). After adjusting for age, BMI, cumulative duration of cART use and the use of PI and NNRTI, the OR for developing a metabolic event was statistically significantly higher in patients with normal versus decreased ITPase activity (3.11, 95% CI 1.34–7.21; p=0.008) in abacavir-containing regimens. In regimens containing tenofovir or didanosine, no association between ITPase activity and the occurrence of metabolic events could be demonstrated (Figure 1a). Also in regimens without a purine analogue but in 60% containing a PI no association was found (Figure 1a).

In regimens prescribed in patients with wt/wt genotype, metabolic events were more frequently found compared with the other genotypes (537/960 regimens versus 202/435 regimens; p=0.001) (Table 3). Metabolic events occurred more frequently in patients using abacavir and having the wt/wt genotype (p=0.003). This increase could not be found in patients using tenofovir or didanosine (Table 3). However, after adjusting for age, BMI, cumulative cART use and the use of PI or NNRTI, no significant difference in metabolic events between patients using abacavir with wt/wt genotype and the other *ITPA* genotypes could be demonstrated (Figure 1b).

DISCUSSION

In this study we show, for the first time, that ITPase activity may play a role in the association of abacavir use and the occurrence of metabolic events in HIV-infected patients using cART. In regimens containing abacavir decreased ITPase activity was associated with fewer metabolic adverse events. We could not confirm the hypothesis that decreased ITPase activity decreased the risk of CVD. No association between *ITPA* genotype and CVD or metabolic events was found after adjustment for other CVD risk factors.

In multiple studies it was reported that the use of abacavir increased the risk of developing CVD or myocardial infarction.[9–11] Other studies, however, could not confirm this association.[14,25] We found no association between abacavir use, ITPase activity and CVD events, possibly owing to the relatively small number of CVD events found in our study. Like oth-

ers, we found that metabolic events were associated with abacavir, but not with tenofovir use.[26–30] A possible explanation may be the difference in chemical structure since tenofovir is an adenosine analogue and abacavir is a guanosine analogue.

How ITPase might be involved in the occurrence of metabolic events during abacavir use is not known. Carbovir triphosphate was found not to be a direct substrate for ITPase.[20] Potentially the answer lies in cellular signal transduction. Guanosine 3',5'-cyclic monophosphate (cGMP) is an important secondary messenger that modulates multiple cellular processes, such as platelet aggregation, neurotransmission, blood pressure, lipolysis and gut peristalsis.[31,32] cGMP is produced from GTP by the enzyme soluble guanylate cyclase (sGC)[33,34] and stimulation of sGC was found to be protective against obesity.[35] However, in the presence of nitric oxide (NO) and magnesium ions (Mg^{2+}) sGC shifts its substrate specificity to ITP to form inosine 3',5'-cyclic monophosphate (cIMP) and also, in the absence of hypoxaemia, addition of exogenous ITP to intact porcine arteries led to higher cIMP levels.[36,37] In contrast to cGMP, which induces vasodilatation, cIMP induces vasoconstriction.[36] This vasoconstriction has been well studied by measurement of flow-mediated dilatation (FMD) of the brachial artery and is strongly associated with the risk of CVD.[38–40] Patients using abacavir were found to have lower FMD than patients not using abacavir,[41] whereas didanosine and tenofovir use were not associated with lower FMD. Support for the hypothesis that abacavir shifts sGC to use ITP instead of GTP, leading to higher cIMP levels, can be found in the study by Baum et al.[42] Formation of cGMP was decreased in human platelets incubated with carbovir triphosphate (the active metabolite of abacavir) and the authors concluded that carbovir triphosphate inhibited sGC. The question remains whether sGC was inhibited, carbovir triphosphate competed with GTP or a shift in substrate specificity of sGC occurred. New research is warranted to investigate under which circumstances ITP is a substrate for sGC, what the consequences of this shift in substrate specificity are and what the impact of carbovir triphosphate is on sGC activity in human cells.

In 2010 Fellay et al.[17] showed that the SNPs 94C>A and 124+21A>C in the *ITPA* genotype were associated with the protection against hemolytic anemia during treatment with ribavirin for hepatitis C infection. We found ITPase might be a more accurate predictor of the development of hemolytic anemia in these patients than *ITPA* genotype.[43] In HIV-infected patients both ITPase activity and *ITPA* genotype were associated with adverse events; however, *ITPA* genotype correlated less well with adverse events compared with ITPase activity.[20] In the present study again, we found *ITPA* genotype and ITPase activity were both crudely associated with adverse events; however, after adjusting with the logistic mixed effects model, only ITPase activity remained statistically significant. The difference in the effect of ITPase activity versus *ITPA* genotype in HIV-infected patients may be explained by the finding that in these patients the wt/wt and the c.94C>A carriers have decreased

erythrocyte ITPase activity compared with non-HIV-infected patients carrying the same genotypes.[19]

Here, we report a lower risk of metabolic events in HIV-infected patients that use abacavir and have decreased ITPase activity. In a previous study, in tenofovir-containing regimens, decreased ITPase activity was also associated with a lower risk of adverse events.[20] In contrast, the use of abacavir in this previous study was crudely associated with an increase in adverse events in patients having decreased ITPase activity. The adverse events in that study were different from the current study and included gastro-intestinal, neurological, renal, skin and liver-related adverse events, and potentially this explains why decreased ITPase activity may lead to an increase in adverse events in one study, but a decrease in adverse events in the other study. In other studies the association between decreased ITPase activity or a SNP in the *ITPA* genotype and adverse events was shown to be dependent on the kind of adverse event and the drug that was used, leading to, for instance, an increase in hepatic toxicity (during azathioprine or 6-mercaptopurine use) but a decrease in hemolytic anemia (during ribavirin use).[44–47] We hypothesize that the effect of ITPase activity in patients using abacavir is also dependent on the nature of the adverse events.

There are limitations that need to be mentioned. Our study was retrospective with all the possible caveats of retrospective studies. For example, factors other than the cART regimen could have contributed to alterations in metabolic events. This is, however, not different for patients with decreased or patients with normal ITPase activities, and thus the influence of this factor seems limited. Although our results could be mechanistically made plausible, further prospective studies are still warranted to confirm our results. Owing to the number of patients included, a low frequency of cardiovascular events was found. This may explain the lack of association of CVD with ITPase activity. Also, the clinical relevance of our findings is to be further established by larger prospective trials.

In conclusion, we showed that ITPase activity may be associated with a risk of metabolic events in HIV-infected patients using abacavir. Using ITPase activity as a potential biomarker to predict adverse metabolic events may be a further step towards more patient-tailored medicine in the future. However, elucidation of the pathogenic mechanism needs further studies and our results need to be confirmed in a prospective trial.

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Chapter 7.

Persistent metabolic changes in HIV-infected patients during the first year of combination antiretroviral therapy.

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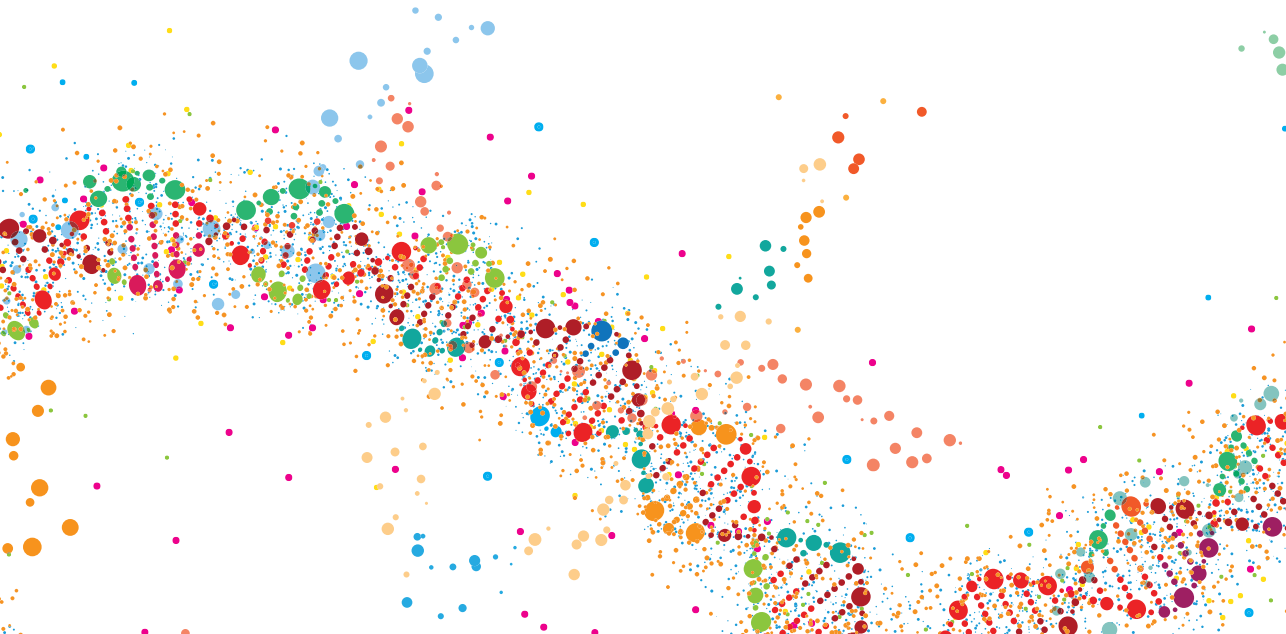

ABSTRACT

The HIV-human metabolic relationship is a complex interaction convoluted even more by antiretroviral therapy (cART) and comorbidities. The ability of cART to undo the HIV induced metabolic dysregulation is unclear and under-investigated. Using targeted metabolomics and multiplex immune biomarker analysis, we characterized plasma samples obtained from 18 untreated HIV-1-infected adult patients and compared these to a non-HIV infected (n = 23) control population. The biogenic amine perturbations during an untreated HIV infection implicated altered tryptophan- nitrogen- and muscle metabolism. Furthermore, the lipid profiles of untreated patients were also significantly altered compared to controls. In untreated HIV infection, the sphingomyelins and phospholipids correlated negatively to markers of infection IP-10 and sIL-2R whereas a strong association was found between triglycerides and MCP-1. In a second cohort, we characterized plasma samples obtained from 28 HIV-1-infected adult patients before and 12 months after the start of cART, to investigate the immune-metabolic changes associated with cART. The identified altered immune-metabolic pathways of an untreated HIV infection showed minimal change after 12 months of cART. In conclusion, 12 months of cART impacts only mildly on the metabolic dysregulation underlying an untreated HIV infection and provides insights into the comorbidities present in virally suppressed HIV patients.

INTRODUCTION

The interaction of the Human immunodeficiency virus (HIV) with its host is a complex process with a growing body of literature revealing the capacity of HIV to induce a plethora of metabolic changes in the human body.[1-5] Hypertriglyceridemia was identified as one of the first metabolic consequences of HIV infection.[6] Since then, studies using Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) applied to biofluids of HIV-infected individuals have confirmed the presence of HIV induced metabolic alterations.[1,7-9] Investigating *in vitro* models of HIV infection using primary macrophages and $CD4^+$ T-cells had different metabolic outcomes during HIV infection. $CD4^+$ T-cells infected with HIV exhibited increased glucose uptake and upregulated glycolytic intermediates compared to reduced glucose uptake and steady-state glycolytic intermediates in HIV-infected macrophages.[10] While, *in vivo* models using rhesus macaques infected with simian immunodeficiency virus (SIV) revealed increased fatty acids, phospholipids, and acyl-carnitines, suggesting an impaired mitochondrial fatty acid oxidation.[11] Comparatively, serum and plasma derived from HIV infected individuals revealed altered metabolites of lipid and mitochondrial pathways as well as organic acids and fatty acids.[1,5] Moreover, the saliva from HIV-infected patients versus healthy controls revealed alterations in carbohydrate biosynthesis and degradation.[2]

Infection with HIV causes a progressive malfunctioning of the human immune system. HIV infection depletes $CD4^+$ T-cells, while also inducing functionally exhausted $CD8^+$ T-cells and impaired NK cells, leaving the host vulnerable to opportunistic infections.[12] Furthermore, the metabolic and immunological changes in untreated HIV patients increase the risk of developing comorbidities, including cardiovascular disease (CVD), insulin resistance and HIV-associated neurocognitive disorders (HAND).[13,14]

The advent of combination antiretroviral therapy (cART) provided an important lifeline for HIV patients, since cART effectively inhibits HIV replication to undetectable levels, while also enabling the restoration of the immune system with increasing $CD4^+$ T-cell counts. Nevertheless, important metabolic-related side-effects of cART are reported in patient populations, mainly lipodystrophy and insulin resistance, which may further predispose virally suppressed patients to increased CVD, diabetes, and kidney damage.[15-17] Also, studies have shown that although cART dramatically decreased the incidence of the most severe clinical phenotype of HAND (HIV associated neurocognitive disorder) – HIV-associated dementia – in virally suppressed patients the milder forms of HAND – asymptomatic neurocognitive impairment and mild neurocognitive disorders – have become more prevalent.[18,19] One aspect of the pathogenesis of CVD or HAND in successfully treated HIV patients is that while cART effectively suppresses HIV replication and activity, low levels of immune activation are sustained.[20-22] The ability of cART to rectify the HIV-induced metabolic dysregulation

is unclear and under-investigated. However, this may be highly relevant since persistent metabolic stress could be an underlying pathogenic mechanism in the comorbidities of untreated HIV patients as well as cART suppressed HIV patients. Robust characterization of the metabolic alterations experienced during HIV infection is needed to determine the effect of cART on these pathways, in cART suppressed HIV patients.

To study this, we used comprehensive targeted metabolomics techniques integrated with classical immunological assays and compared changes in plasma of untreated HIV-1 patients to non-HIV infected individuals, and paired plasma of untreated HIV-1 patients at baseline to their plasma after 12 months of cART in the HIV-suppressed state, to assess whether profiles normalized to the situation in healthy individuals or remained perturbed. We found dysregulated biogenic amine and lipid metabolism in untreated HIV-infected patients, conditions that have been independently associated with the pathophysiology of CVD and HAND. After 12 months of cART, metabolic changes were found for some biogenic amines, while the lipid metabolites revealed increasing levels in the virally suppressed patients.

METHODS

Patients and sample collection

Untreated HIV versus non-HIV-infected controls, population A

For the comparison of metabolic profiles of untreated HIV-1-infected patients to a non-HIV infected control population, 18 HIV-1-infected, cART naïve patients were selected from the outpatient clinic of the Erasmus Medical Center in Rotterdam, The Netherlands, archived sample bank. The plasma was stored at −80°C. Inclusion criteria were age over 18 years and no previous treatment for HIV. Exclusion criteria were severe comorbidity (e.g. diabetes mellitus, cardiovascular disease, opportunistic infection), coinfection with hepatitis B or C, use of alcohol > 2 IU/day and use of co-medication. All patients had given written informed consent for inclusion in the database of the Dutch HIV monitoring foundation (Stichting HIV Monitoring; SHM) also known as the AIDS Therapy Evaluation in the Netherlands (ATHENA) cohort, and collection of demographic, laboratory and clinical data from the medical records and storage and future use for scientific research of biological material. Their plasma samples were compared to a non-HIV infected control population from volunteers (n = 23), without comorbidity or co-medication, which had all given their written informed consent.

Untreated HIV versus HIV-suppressed (12 months of cART), population B

For the comparison of metabolic profiles of untreated HIV-1-infected patients to the HIV-suppressed situation, plasma samples of 28 HIV-1-infected patients from the outpatient

clinic of the Maastricht University Medical Center in Maastricht, The Netherlands were selected in the archived sample bank. The plasma was stored at −80°C. All patients had given written informed consent for inclusion in the database of the Dutch HIV monitoring foundation and collection of demographic, laboratory and clinical data from the medical records and storage and future use for scientific research of biological material. From each included patient, a plasma sample was selected from before the start of cART and at 12 months after the start of cART. All patients were older than 18 years at the start of cART and had not received any previous ART therapy. All patients started with a cART regimen containing Abacavir. The patients had no diabetes mellitus and no diagnosed autoimmune diseases. We retrieved information regarding dyslipidaemia from the earlier laboratory results prior to the 12 months samples after the start of cART. Because the patients were generally not in care prior to the HIV diagnosis, no values were available from the blood sample prior to the start of cART. The study was performed according to the Helsinki Declaration and approved by the Ethical Committee of the Maastricht University Medical Center.

Targeted LC-MS metabolomics

Targeted metabolomics analyses were performed using standard operating procedures derived from previously published methods.[23-26] Detailed procedures and target lists are provided in the Additional file 1 - Methods with a brief overview of the four platforms used given in Table 1. After LC-MS analyses, peak integration was done using the instrumental software, and the relative ratios between metabolites and their corresponding internal standards were determined.

Metabolomics quality controls

Quality control (QC) samples consisted of equal aliquots of a QC pool made by combining equal volumes (±25 µL) of all study samples. A set of QC samples was then included during the analyses of the experimental groups on the individual metabolomic platforms and evenly distributed across the randomized samples prior to LC-MS analyses. In addition, independent duplicate samples (10–15%) were randomly selected. Using the QC samples and duplicate samples, a double-QC approach was applied to include metabolites that were reliably measured by the individual metabolomics platforms by reporting and using only those metabolites for which both duplicate samples and QC samples showed an RSD < 30%.

Multiplex immunoassays to assess plasma biomarkers

The levels of cytokines, chemokines, growth factors and other proteins were determined using the Procarta Plex human Immune Monitoring Panel (Affymetrix, Vienna, Austria). The panel measured 14 proteins simultaneously and consisted of IP-10, IL-10, IL-6, D-dimer, sIL-2R, IL-21, IL-18, MCP-1, sPD-L1, sPD-L2, sTIM-3, sPD1, CRP, and sTNF-RII. The assay was conducted according to the manufacturer's instructions, identical to the procedures

Table 1. Metabolomics platforms. A brief overview of the platforms detailing volumes, sample preparation and analytical instruments.

Targeted metabolomics platform	Volume serum used	Sample preparation method	Analytical platform	Metabolite class coverage	Platform targets, n		
					Total	Quality control passed	% Missing data
Biogenic amine[24]	5 µL	Protein precipitation & AccQ-Tag derivatization	UPLC[a]-TQMS[b]	Amino acids, catecholamines & polyamines	100	62	0%
Positive lipid[23]	10 µL	Isopropyl alcohol extraction	UPLC[a]-QToF[c]	Lysophospholipids, phospholipids, cholesterol esters, di/triglycerides & spingomyelins	250	147	0.02%
Oxylipins[25]	250 µL	Oasis HLB SPE extraction	HPLC[d]-MS/MS[e]	Hydroxylated fatty acids, prostaglandins & thromboxanes	120	68	7.7%
Oxidative stress[26]	150 µL	Butanol:Ethyl acetate liquid-liquid extraction	UHPLC[f]-MS/MS[e] Low pH run	Isoprostanes, nitro-fatty acids, sphingosine & sphinganine	46	36	6.6%
			UHPLC[f]-MS/MS[e] High pH run	Sphingosine-1-phosphate & lysophosphatidic acids species			

[a] UPLC, Ultra Pressure Liquid-Chromatography; [b] TQMS, Triple quadrupole mass spectrometer; [c] QToF, Quad Time of Flight; [d] HPLC, High-Pressure Liquid-Chromatography; [e] MS/MS, Triple quadrupole Mass Spectrometer; [f] UHPLC, Ultra High-Pressure Liquid-Chromatography.

used in our previous studies.[27-29] The concentrations of analytes were measured using the microsphere-based multiplex Luminex-100 (Luminex Corporation, Austin, TX, USA). Samples to compare untreated HIV with non-HIV controls were analyzed in a different run than paired samples of untreated and HIV-suppressed patients. Data were analyzed using ProcartaPlexAnalyst 1.0 (www.ebioscience.com/resources/procartaplex-analyst-1.0-software.htm).

Statistical analyses

SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for Fisher's exact tests on the patient cohort characteristics presented as frequencies, and ANOVA on the continuous values. A combination of univariate and multivariate bioinformatics approaches was performed using the R script–based online tool Metaboanalyst 3.0, a comprehensive tool suitable for analysing metabolomics data.[30] The metabolomics datasets were log transformed and auto-scaled prior to bioinformatics analyses. For the analyses between controls and untreated HIV patients, significant metabolites were identified per metabolomics platform based on the following criteria: i. a p-value < 0.05 using the unpaired student t-test, and ii. a fold change (FC) ≥ 1.30 or ≤ 0.70, indicating a 30% increase or decrease. The False discovery

rate's q-values are reported for every reported p-value. For the analyses between the paired untreated HIV patients and 12 months cART follow up, significant metabolites were identified per metabolomics platform based on a False discovery rate adjusted p-value < 0.05 using a paired student t-test. Plasma protein biomarkers were analysed using the unpaired student t-test for comparing controls and untreated HIV patients, and the paired student t-tests across the paired patients' samples at baseline and 12 months. GraphPad Prism 7 software was used in the rendering of graphs and figures.

Two types of immune-metabolic correlation networks were done. Firstly, for the control group and the untreated HIV patient group, independent Spearman correlation analyses were done using the absolute metabolite and cytokine levels. Next, per group cytokine levels were correlated with each other to form the network skeleton. The second type of correlation network was based on Spearman correlations of the levels of metabolites to cytokines in HIV-suppressed patients after 12 months of cART relative to the levels at baseline from paired patient samples (relative change = 12 months cART – baseline). Next, we correlated the relative change in cytokine levels to form the network skeleton. For both types of immune-metabolic correlations significant correlations were defined using a cut-off p-value (two-tailed) < 0.01 and were visualized as a network using the Metscape application within Cytoscape (v3.4.0).

Ethics approval and consent to participate

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. The ethical review board of the Erasmus MC has approved the study, and informed consent was obtained from all patients who were asked to donate blood.

RESULTS

Untreated HIV versus non-HIV-infected controls

Patient population A

Most of the individuals in both the patient and the control populations listed in Table 2 were male and Caucasians. Mean age did not differ between both groups. The mean $CD4^+$ T-cell count of the HIV-1 patients was 441×10^6 cells/L with an interquartile range (IQR) of 367.5×10^6. The untreated HIV patients had a large heterogeneity in viral load with a median of 1.11×10^5 viral copies and an IQR of 2.64×10^5 especially when taking the minimum and maximum values as listed in Table 2 into account.

Table 2. Characteristics of the untreated HIV-infected patients (Population A) and the non-HIV infected control population

Characteristics	Patients (n=18)	Controls (n=23)	P-value
Age; mean years ± SD[a]	40 ± 10	36 ± 10	0.18
Male Gender, n (%)	16 (88.9)	14 (60.9)	0.08
Caucasian, n (%)	14 (77.8)	17 (73.9)	0.90
Mean $CD4^+$ T-cell count; $x10^6$/L ± SD[a]	441 ± 265	-	-
HIV viral load; median copies/mL (range)	1.1 $x10^5$ (60-$1x10^7$)	-	-

[a] SD, Standard deviation

Altered plasma metabolic profiles in untreated HIV-infected patients

To characterized metabolic alterations experienced during an untreated HIV-1 infection, we compared 18 untreated HIV-1-infected patients (population A) with 23 controls through profiling their biogenic amines, lipids, and signalling lipid metabolites. As shown in the volcano plot in Fig. 1a, 18 biogenic amines were differently affected in HIV patients versus controls, with reduced levels in all (see Supplementary Table S1). Reduced levels of antioxidants, including total glutathione and taurine, together with lipid headgroup moieties o-phosphoethanolamine and the choline metabolite sarcosine were identified as the most significantly altered amine metabolites during an untreated HIV infection. Furthermore, untreated HIV patients had decreased levels of tryptophan and serotonin compared to controls. The plasma kynurenine/tryptophan (K/T) ratio, as a readout for indoleamine 2,3-dioxygenase (IDO) activity, showed upregulated IDO activity during untreated HIV infection compared to controls ($p = 0.0003$) (see Supplementary Fig. S1A). Concurrently investigating the plasma serotonin/tryptophan ratio reflected the consequences of increased IDO activity for serotonin synthesis during untreated HIV infection compared to controls ($p < 0.0001$) (see Supplementary Fig. S1B). Decreased levels of the branch chain amino acids leucine and isoleucine as well as carnosine reflected impaired muscle metabolism during an untreated HIV infection. The significantly decreased levels of ornithine and putrescine in untreated HIV patients also hints at a reduced urea cycle and thus ammonia clearance in these patients compared to controls. Additionally, decreased levels of alanine, asparagine, α-aminobutyric acid, tyrosine, and methionine were also detected in untreated HIV patients compared to controls. Evaluation of the levels of plasma lipids, which included the different lysophospholipids classes, phospholipids, di/triglycerides, sphingomyelins, ceramides, and cholesterol esters, revealed 44 modulated metabolites with a $p < 0.05$ and a fold-change (FC) ≥ 1.30 or ≤ 0.70 (Fig. 1b and Supplementary Table S2). The lysophospholipids class presented with 6 lysophosphatidic acid (LPA) species having increased plasma levels in untreated HIV-infected patients versus controls, and one lysophosphatidylcholine (LPC) metabolite which was reduced compared to controls. Furthermore, two monounsaturated sphingomyelin (SM) species, two PUFA cholesterol ester (CE), and two long-chain saturated

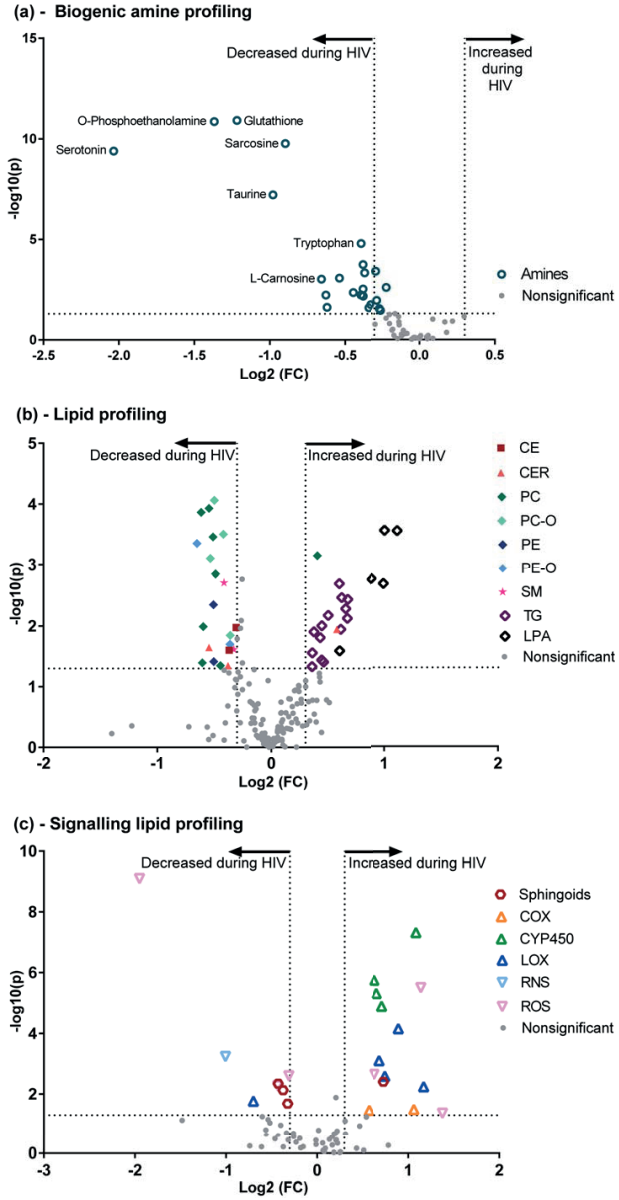

Figure 1. Plasma metabolic characterization of untreated HIV vs controls. Volcano plot of (a) the biogenic amine profile, (b) the lipid profile and (c) the signalling lipid profile. Volcano plots are representative of the -log10 (Mann-Whitney p-value) on the y-axis with the x-axis showing the log2 (Fold change) of the metabolite, between the controls and the untreated HIV patients (population A) respectively. Dashed lines represent the respective significance thresholds, with the significant metabolites identifiable by coloured symbols with either a name or corresponding class colour identifier. CE, Cholesterol esters; CER, Ceramides; PC, Phosphatidylcholines; PC-O, Plasmalogen PCs; PE, Phosphatidylethanolamines; PE-O, Plasmalogens PEs; SM, Sphingomyelins; TG, Triglycerides; LPA, Lysophosphatidic acid; COX, Cyclooxygenase; CYP450, Cytochrome P450; LOX, Lipoxygenase; RNS, Reactive nitrogen species; ROS, Reactive oxygen species.

ceramides (CER) metabolites all had reduced levels in untreated HIV patients. The triglycerides (TG) profile presented with 14 mostly polyunsaturated triglycerides species showing significantly increased levels during untreated HIV infection. The phospholipids presented with seven phosphatidylcholines (PC), four plasmalogen phosphatidylcholines (PC-O), two phosphatidylethanolamines (PE) and two plasmalogen phosphatidylethanolamines (PE-O) all having decreased levels during untreated HIV.

The third subclass of metabolites that were evaluated for differences between untreated HIV patients and controls was the class of signalling lipid mediators, derived from the enzymatic or free radical oxidized polyunsaturated fatty acids, which includes the prostaglandins, thromboxanes, hydroxy-fatty acids, leukotrienes, resolvins, epoxy-fatty acids, isoprostanes and nitro fatty acids. During the untreated HIV infection 22 significantly affected signalling lipid metabolites were identified of which 8 had reduced levels and 14 had increased levels in the untreated HIV patients compared to controls. (Fig. 1c, Supplementary Table S3). The four signalling mediators: 5,6-DiHETrE, 8,9-DiHETrE, 11,12-DiHETrE, and 14,15-DiHETrE all derived from arachidonic acid and synthesised by cytochrome P450 isozymes showed the most significant increased levels in the untreated HIV patients compared to the controls. Furthermore, untreated HIV patients had increased levels of dihydrosphingosine but decreased levels of three different Sphingosine-1-phosphate species compared to controls. The metabolites 5-HETE, 12-HETE and 15-HETE derived from the Lipoxygenase activity on Arachidonic acid, also had increased levels compared to the controls. Oxidized lipids due to the activity of ROS and RNS showed differential responses with increased levels of 8-iso-PGE2, 2,3-dinor-PGF2a and 20 HETE and decreased levels of 11-HDoHE and 8,12-iPF2α-IV in untreated HIV patients compared to controls.

Untreated HIV versus HIV-suppressed (12 months of cART)

Patient population B

The characteristics of the HIV-1-infected patients selected to compare baseline metabolic profile to the HIV-suppressed profile after 12 months of therapy (population B) are presented in Table 3. This is an independent population from the untreated HIV-infected population A introduced in Table 2. Most of the patients had a male gender. Mean $CD4^+$ T-cell count at the start of cART was of 267×10^6 cells/L with an IQR of 224×10^6. There was a large heterogenicity in viral load with a median of 5.5×10^4 copies/ml and an IQR of 2.7×10^5. 89% of the patients were of European descent. Four patients had a blip in their viral load at 12 months on cART: their viral loads were 53, 73, 103 and 130 copies/ml. Most patients used Abacavir in combination with Lamivudine as the backbone in cART (n = 25). The patients who only used NRTI's all used the combination Abacavir with Lamivudine and Zidovudine (n = 8) except for one patient who used Abacavir/Lamivudine with Stavudine. The protease inhibitors (PI) used were Lopinavir (in combination with ritonavir) (n = 4), Atazanavir

Table 3. Characteristics of 28 HIV-infected patients (Population B) at baseline and after 12 months of cART

Characteristics	At baseline	12 months of cART[a]
Age; mean years ± SD[b]	40 ± 10	
Male Gender, n (%)	21 (75.0)	
European region of origin, n (%)	25 (89.3)	
Statin use, n (%)	4 (14.3)	5 (17.9)
Mean Cholesterol[c] >6.5[d], n (%)	unknown	6 (21.4)
Mean LDL >4.5[d], n (%)	unknown	4 (14.2)
Mean Triglycerides >2.3[d], n (%)	unknown	8 (28.6)
Mean BMI[c], kg/m^2 ± SD[b]	22.7 (3.1)	23.1 (3.3)
Hepatitis B coinfection, n (%)	1 (3.6%)	
Hepatitis C coinfection, n (%)	8 (28.6%)	
Alcohol ≥5 IE/day, n (%)	1 (3.6%)	
Hard drugs[f]/methadone use, n (%)	8 (28.6%)	
Mean $CD4^+$ T-cell count[h], $x10^6/L$ ± SD[b]	267.4 (158.9)	489.0 (209.4)
Mean time on cART, months ± SD[b]	n.a.	12.5 (1.2)
HIV viral load <10^2 [h], n (%)	0 (0%)	26 (93.6%)
HIV viral load >10^2 [h], n (%)	28 (100%)	2 (7.2%)
Median HIV viral load[h] (range)	$5.5x10^4$ ($1.6x10^3$ – $8.1x10^5$)	
cART regimen		
NRTI[i] + NNRTI[j], n (%)	n.a.	13 (46.4%)[l]
NRTI[i] + PI[k], n (%)	n.a.	6 (21.4%)[l]
NRTI[i] only (n/%)	n.a.	9 (32.1%)[l]

[a] cART, combination Anti-retroviral therapy; [b] SD, Standard deviation; [c] mean value of 6 months prior to sample used; [d] mmol/L; [e] BMI, Body mass index; [f] cocaine, heroin and/or MDMA (3,4-methylenedioxymethamphetamine); [g] $x10^6/L$; [h] copies/ml; [i] NRTI, Nucleoside reverse transcriptase inhibitor; [j] NNRTI, Non-nucleoside reverse transcriptase; [k] PI = Protease inhibitor; [l] Three patients used a different cART regimen at 6 months versus 12 months of cART. Because of one patient switching from a protease inhibitor (PI) based regimen to a non-nucleoside reverse transcriptase inhibitor (NNRTI) or nucleoside reverse transcriptase inhibitor (NRTI) only regimen and vice versa, the proportion of PI-based, NNRTI based or NRTI the only regimen remained unchanged in the entire group.

(n = 1) and Darunavir (n = 1). The NNRTI's used were Efavirenz (n = 8), Nevirapine (n = 4) and Rilpivirine (n = 1). Five patients used a statin. The incidence of dyslipidaemia or the use of lipid-lowering therapy (statins and/or fibrates) remained stable during the first year of cART.

Metabolic consequences following cART treatment of HIV patients

Next, we evaluated the metabolic changes associated with 12 months of cART treatment, as well as HIV suppression in the longitudinal patient population B. cART treatment of the HIV patients resulted in viral suppression with undetectable plasma HIV RNA levels and partial restoration of $CD4^+$ T-cell counts in the blood (Table 3). Eight HIV infected

patients were HCV positive, but were untreated for HCV during the 12-month period and were not excluded from the analyses. The untreated HCV infection had minimal impact on the metabolic findings as can be seen in see Supplementary Fig. S2. Evaluation of biogenic amine levels in paired plasma samples showed significantly increased methionine sulfone, histidine and tryptophan levels during the therapy period as compared to the baseline samples (untreated HIV), whereas pipecolic acid, 3-aminoisobutyric acid, and kynurenine showed significantly decreased levels during the therapy period as compared to the baseline (Fig. 2a, Supplementary Table S4). Significant metabolites were identified by having a false discovery rate adjusted p-value < 0.05 using a paired t-test approach. The K/T ratio revealed significantly decreased IDO activation after 12 months of cART (see Supplementary Fig. S3), however, the ratio between tryptophan and serotonin showed no significant changes. The neurotransmitter dopamine and metabolite α-aminobutyric acid correlated with $CD4^+$ T-cell counts during 12 months of therapy (see Supplementary Fig. S4A).

The lipid profile showed an overall increasing trend reflecting higher levels after the 12-month cART period compared to their baseline levels, as can be seen in Fig. 2b. Predominantly it was the phosphatidylcholine metabolism responding to cART with significantly increased levels of 9 phosphatidylcholine species, 8 plasmalogen phosphatidylcholine, and 3 lysophosphatidylcholine species after 12 months of cART compared to baseline values prior to starting of cART. Furthermore, 7 sphingomyelin metabolites, together with one phosphatidylethanolamine species, two plasmalogen phosphatidylethanolamine, and a cholesterol ester were also increased after 12 months of cART compared to their baseline values. Furthermore, the phosphatidylcholines also revealed a positive association with $CD4^+$ T-cell counts of the patients (see Supplementary Fig. S4B).

Finally, we compared changes in the signalling lipid profiles in patient plasma after 12 months of cART to their untreated HIV levels and found no significant changes.

Plasma immune-metabolic networks of HIV infection and therapy

Plasma levels of cytokines and immune mediators are a commonly used readout reflecting systemic immune activity.[31,32] To correlate metabolite profiles in plasma with immune activity, we determined in subsequent plasma samples of the same patients the levels of the infection markers IP-10, sIL-2R, and D-dimer, the inflammation markers IL-6, IL-10, IL-21, IL-18, and MCP-1, and the soluble exhaustion markers sPD1, sPD-L1, sPD-L2, and sTIM-3. Significantly enhanced plasma levels of D-Dimer, IL-18, MCP-1, sPD-L2, sTIM-3, IP-10, sIL-2R, CRP, and sTNF-RII were observed in patients with untreated HIV as compared to non-HIV infected controls (Population A), and are shown in Supplementary Fig. S5. The other mediators tested were below the limit of detection of the assay, which included IL-6, IL-10, IL-21, sPD1, and sPD-L1.

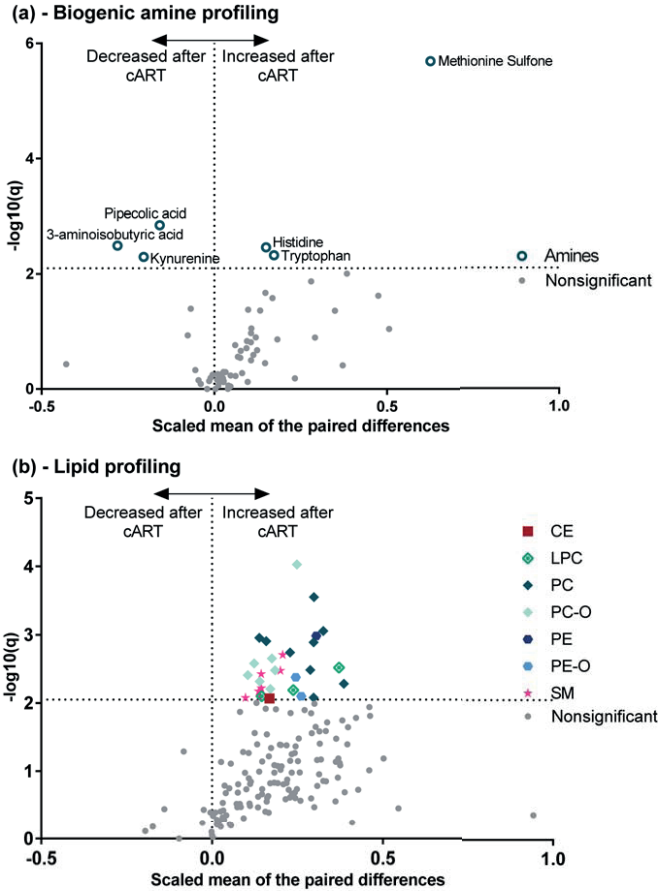

Figure 2. Plasma metabolic characterization of response to 12 months' cART. Modified volcano plots showing (a) the biogenic amine profile, and (b) the lipid profile. The modified volcano plots are representative of the -log10 (FDR adjusted paired T-test p-value) on the y-axis with the x-axis showing the scaled mean of the paired differences per metabolite, between the baseline and 12 months cART follow-up sample (patient population B). The horizontal dashed line represents a false discovery rate adjusted q-value <0.05. Significant metabolites are identifiable by coloured symbols with either a name or corresponding class colour identifier. CE, Cholesterol esters; LPC, Lysophosphatidylcholines; PC, Phosphatidylcholines; PC-O, Plasmalogen PCs; PE, Phosphatidylethanolamines; PE-O, Plasmalogens PEs; SM, Sphingomyelins.

Evaluating the changes in the immunological parameters in population B after the 12 months cART period revealed an immunological picture of effective therapy shown in Supplementary Fig. S6. Markers of infection revealed that after the initiation of cART, both plasma IP-10 and sIL-2R levels were reduced significantly. Furthermore, initiation of cART reduced the levels of the inflammation marker IL-18 whereas MCP-1 levels remained unchanged when comparing baseline to 12 months of cART. An improvement in markers of exhaustion was found with reducing levels of both sPD-L2 and sTIM-3 after 12 months of cART.

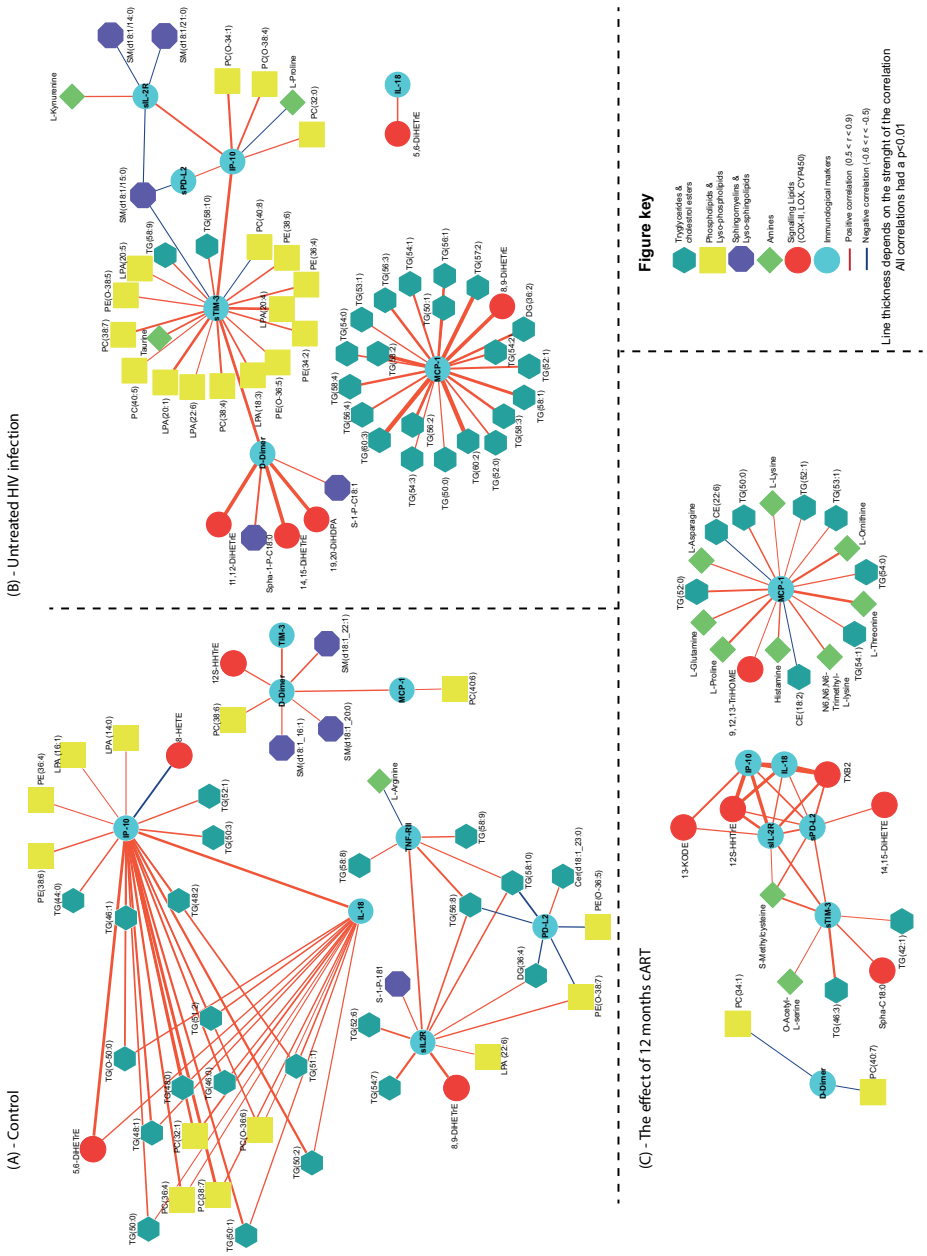
(A) - Control
(B) - Untreated HIV infection
(C) - The effect of 12 months cART
Figure key
Tryglycerides & cholesterol esters
Phospholipids & Lyso-phospholipids
Sphingomyelins & Lyso-sphingolipids
Amines
Signalling Lipids (COX-/-, LOX, CYP450)
Immunological markers
Positive correlation (0.5 < r < 0.9)
Negative correlation (-0.6 < r < -0.9)
Line thickness depends on the strenght of the correlation
All correlations had a p<0.01

Figure 3. Immune-metabolic networks of controls, untreated HIV and the influence of cART. (A) Control immune-metabolic correlation network based on correlating the absolute levels of plasma metabolites and plasma cytokines in the control group. (B) Untreated HIV immune-metabolic correlation network based on correlating the absolute levels of plasma metabolites and plasma cytokines in the untreated HIV group (Population B). (C) The immune-metabolic correlation network following 12 months cART. Using the paired samples of population B, the relative change in levels of 12 months' cART compared to their baseline levels (untreated HIV sample) were determined for plasma metabolites and cytokines and subsequently correlated. For all three networks, spearman correlations were performed and significant correlations were defined as having a strict p-value (two-tailed) <0.01.

Next, we constructed immune-metabolic correlation networks respectively for the controls and the patients with an untreated HIV infection from population B using the absolute of levels of metabolites and immune mediators. Figure 3 presents these three immune-metabolic correlation networks showing all correlations with a strict p-value cut-off of $p < 0.01$. Comparing Fig. 3a (the control network) to Fig. 3b (the untreated HIV network) difference is seen in the interactions between the two networks. The control network revealed isolated clusters with IP-10 and IL-18 correlating to different lipid classes, sIL-2R and sTNF-RII correlated together and D-Dimer, sTIM-3 and MCP-1 showing correlation. Comparatively the untreated HIV shows positive correlations between sTIM-3, sIL-2R, IP-10 and sPD-L2 with D-Dimer connecting the network through sTIM-3, revealing correlations between markers of infection and exhaustion. In both networks, sTIM-3 and D-Dimer revealed strong positive correlations, while in the untreated HIV patients sTIM-2 also showed strong interactions with phospholipids as well as lysophospholipids. Furthermore, in untreated HIV patients MCP-1 correlated strongly to several triglyceride species compared to the control network where MCP-1 correlate only to D-Dimer. Interestingly, in the control network, IL-18 showed strong interactions with triglyceride species, whereas IL-18 was correlated to only 5,6-DiHETrE in the untreated HIV patients.

Next, for the influence of cART on HIV infection, we constructed immune-metabolic correlation network based on the relative change in levels of metabolites and immune mediators in the paired plasma of the patients on 12 months cART versus their untreated HIV levels. As shown in Fig. 3c, the correlation network revealed distinct immune-metabolic interactions. Firstly, changes in sTIM-3, sIL-2R, IP-10, sPD-L2 and IL-18 positively correlated with each other, with D-dimer becoming disconnected from the core network. Again, MCP-1 showed minimal interactions to other immune mediators in the panel: changes in triglyceride levels correlated strongly to changes in MCP-1 levels as did some amine metabolites, while changes in two cholesterol-esters showed negative correlations. Furthermore, cART diminished the interaction of sTIM-3 with the phospholipids and lysophospholipids which were found in untreated HIV-patients.

DISCUSSION

The data presented in this study provide some insight into the distinct effects of HIV and cART have on metabolism. Through identifying immune-metabolic pathways altered during untreated HIV infection, we can follow these pathways during the first 12 months after commencing cART in the same patients, monitoring their responses. In line with earlier research, we found decreased glutathione, taurine and tryptophan levels as well as upregulated IDO activity in untreated HIV patients compared to HIV-seronegative controls.[8,33,34]

Also, increased levels of highly unsaturated long-chain triglycerides[35] and decreased levels of sphingomyelin species characterized an untreated HIV infection compared to controls. After the initiation of cART, changes in the lipid metabolism were primarily found within the phospholipids while the highly unsaturated triglyceride species remained elevated. A noteworthy metabolic finding is the unresponsive signalling lipid profile, showing minimal changes after the first 12-month cART period. Signalling lipids including eicosanoids are metabolically derived immunological mediators and might represent a metabolic reflection of the immune competence of HIV-infected patients.[36,37] The unresponsive signalling lipid metabolism could result from the sequestering of their polyunsaturated fatty acid precursors into triglyceride species. Cassol *et al*. reported decreased eicosanoid levels specifically 5-hydroxyeicosatetraenoic acid (5-HETE), prostaglandin B2, prostaglandin E2 and thromboxane B2 in cART-treated HIV-positive individuals compared to controls.[1] Since eicosanoids also play an essential role in immunological crosstalk[38] it could help to explain the dysregulated immune surveillance and exhausted phenotype persisting in virally suppressed patients.[39]

Studying the immune-metabolic networks revealed that the initiation of cART disconnected the interactions between kynurenine and sIL-2R as well as sTIM-3's interactions with phospholipids and lysophospholipids. Thus, HIV suppression through cART attenuates the pro-inflammatory sIL-2R and IDO pathways differently, since it has been reported that IDO activity remains significantly increased compared to controls even after 24 months of cART.[40] Secondly, cART might contribute to an exhausted immune phenotype through dysregulating the phospholipids (increased during the first 12 months of cART) and their interactions with sTIM-3 interactions. Further, IL-18, a pro-inflammatory cytokine found elevated in advanced stages of HIV infection,[41] showed strong correlations to triglyceride levels in the control population. The role of IL-18 in cardiovascular disease and the metabolic syndrome, as well as its association with triglyceride levels, has been previously described.[42,43] Comparatively, in the untreated HIV infected population, the triglyceride species showed a strong correlation to MCP-1 levels and not to IL-18. Initiating 12 months of cART was unable to nullify this interaction, revealing that changes in triglyceride levels still strongly correlated to those of MCP-1 in the paired patient samples. Mihăilescu *et al*., found that the prevalence of insulin resistance and metabolic syndrome was higher in HIV suppressed patients compared to virally active patients, and that the levels of MCP-1 correlated to both these co-morbidities,[44] supporting our findings.

Disentangling the mechanism behind the observed metabolic changes during an HIV infection and therapy is a complex task. However, the importance of this task is stressed by the findings in this study that even 12 months of cART does not attenuate metabolic dysregulation. This is opposed to the finding that immune activation in HIV-infected pa-

tients is normalized during treatment with cART for this same period, as we also previously showed.[41] Sustained immune activation thus is not the complete answer to what causes co-morbidity in HIV-infected patients, like neurocognitive disorders and cardiovascular disease. Although cART has become increasingly effective over the last years, but as we show here, is not enough to fully restore the metabolic profile of HIV-suppressed patients to normal.

This finding warrants further research, to contribute to unravelling the metabolic pathophysiological pathways in HIV comorbidities, with multiple findings in this study able to generate testable hypothesis regarding HAND and CVD. With respect to HAND, the following questions arise applicable to both untreated HIV and cART-treated HIV patients. (1) How does the sustained elevated Indoleamine-2,3-dioxygenase (IDO) activity (the rate-limiting enzyme catabolizing L-tryptophan into neurotoxic metabolites and not serotonin) contribute to neurological disorders? (2) What is the pathological mechanism underlying a dysregulated urea cycle during an untreated HIV infection? Since disorders of the urea cycle are known to manifest with neurological implications due to hyperammonemia.[45] Similarly to HAND, questions relating to CVD, an important cause of morbidity and mortality in untreated and suppressed HIV-infected patients, arises.[46,47] (3) Do the significant correlations between changes in triglycerides and MCP-1 levels, expose an alternative mechanism contributing to the development of CVD, independent of IL-18? (4) How does an unresponsive eicosanoid metabolism contribute to CVD, since they play an important role in normal cardiovascular function?[48-50] Potentially with the answer to these questions, morbidity, and mortality in HIV-infected patients because of the non-communicable disease can be further diminished.

It is important to note that in our study the time of follow-up of these patients was too short to draw conclusions about causality. The use of targeted metabolomics platforms in conjunction with other omics technologies would provide a unique opportunity to study HIV pathogenic mechanisms, as well as to identify biomarkers relevant to co-morbidities relating to both HIV and the use of ARVs. Studies investigating the metabolic dysregulation of HIV infections and exposure of antiretrovirals can be further strengthened by investigating paired longitudinal samples with a known outcome. Furthermore, these techniques could also proof complementary to antiretrovirals pharmacokinetic studies currently in clinical trials.

It is important to mention that due to the retrospective nature of the part of our study in which we compared cART naive HIV-infected patients with their samples after 12 months of therapy, it is difficult to ascertain whether all blood samples were processed identically, thereby introducing some variation in outcome parameters. Further, the duration of the

HIV infection prior to starting cART is not known of these patients. Apart from the HIV infection, other factors might play a role in metabolic changes, for instance, smoking habits, aging, and timing of sampling of the material. Also, we have no data on the fasting state of both the patient as well as the control population, however, because the samples of the included patients were taken during routine outpatient visits and blood donors, in general, are instructed not to donate blood in the fasted state, it is safe to assume that both groups were in a post-prandial state. Food intake can affect metabolites and phospholipid levels,[51,52] but also multiple large population studies have shown that changes in plasma lipids and lipoproteins change only modestly during the day, in response to habitual food intake.[53,54] Further, although sub-analysis of 12 months cART according to PI or NNRTI used did not show differences in lipid profiles, the choice of the specific components of the cART regimen might be a significant factor or influence on the metabolic profile of a patient in the long run. For instance, abacavir use, which is associated in the literature with an increase of CVD.[55,56] Population A and B differed significantly from each other regarding mean $CD4^+$ T-cell counts and cytokine profiles, probably due to the fact that Population A was selected to be compared to a non-HIV infected, healthy control population and therefor selection criteria was more strictly defined than those for population B, where the patients were their own controls. In population B, five patients were using statins during the first year of cART, of which four were already receiving statin therapy prior to the start of cART. We were unable to identify any trends in the data indicating that lipid profiles were skewed based on the use of statins. Eight patients were coinfected with HCV, which may have had an effect on the comparison of the untreated patients compared to controls. However, during this study, none of the patients was treated for HCV, thus this was a stable factor in the comparison between untreated HIV and 12 months of cART. A sub-analysis leaving out the HCV coinfected patients showed minimal impact on the outcome. In this study, we were not able to include a sample of the control patients after one year. However, to determine the baseline stability of the metabolic/immune network, further studies are needed with follow-up samples of a control population.

In conclusion, in our study, we found significant changes in the metabolism relevant of untreated HIV infected patients and after 12 months cART. Additional to this finding, and potentially even more important is the finding that cART alone does not restore these changes. Further insight into the metabolic changes caused by HIV infection is warranted to optimize therapy in addition to cART for this patient population.

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AUTHOR CONTRIBUTIONS

A.V., T.H. and A.B. served as principal investigators and conceived and designed the study; J.C.S. and A.C.H. performed the metabolomics experiments; A.B. performed the Luminex analyses; N.C.P., S.H.L., A.V. acquisition of the samples and clinical data; N.C.P., J.C.S., J.B., J.A.B., A.V., T.H., A.B. contributed to study design; J.H. and F.M. performed the statistical analyses. N.C.P. and J.C.S. wrote the first draft of the manuscript with input from all authors, and all authors approved the final manuscript revisions.

AVAILABILITY OF DATA AND MATERIAL

The metabolomics datasets supporting the conclusions of this article will be made available in the online MetaboLights data repository.

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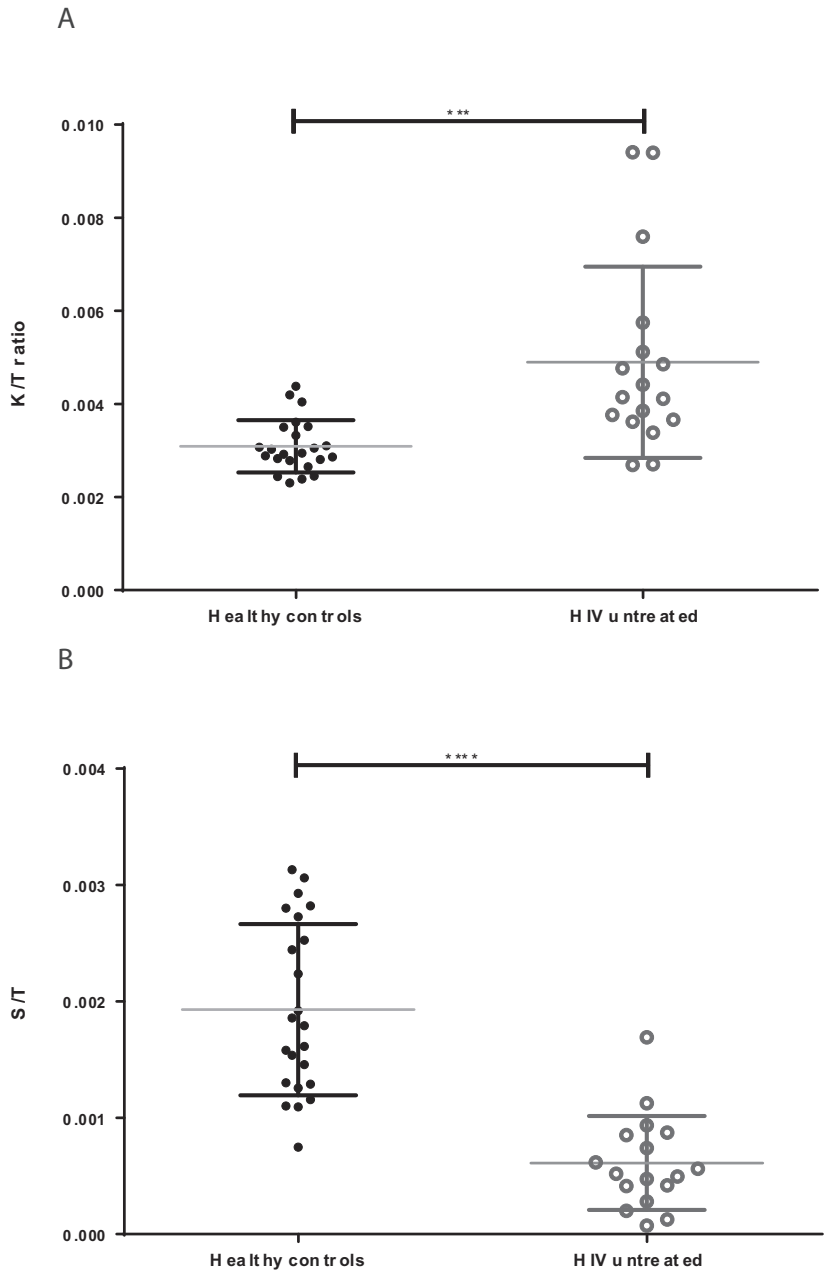


Figure S1: Tryptophan pathway and Indoleamine 2,3-dioxygenase (IDO) activity during an untreated HIV infection. (A) IDO activity during an untreated HIV infection (population A). The kynurenine and tryptophan (K/T) ratio representative of IDO activity are plotted for the controls (Closed dots) and untreated HIV group (Open circles). (B) The serotonin and tryptophan (S/T) ratio showing the altered tryptophan utilization during untreated HIV infection, with the controls (Closed dots) and untreated HIV group (Open circles). Unpaired T-test, *** $p < 0.001$ with **** $p < 0.0001$.

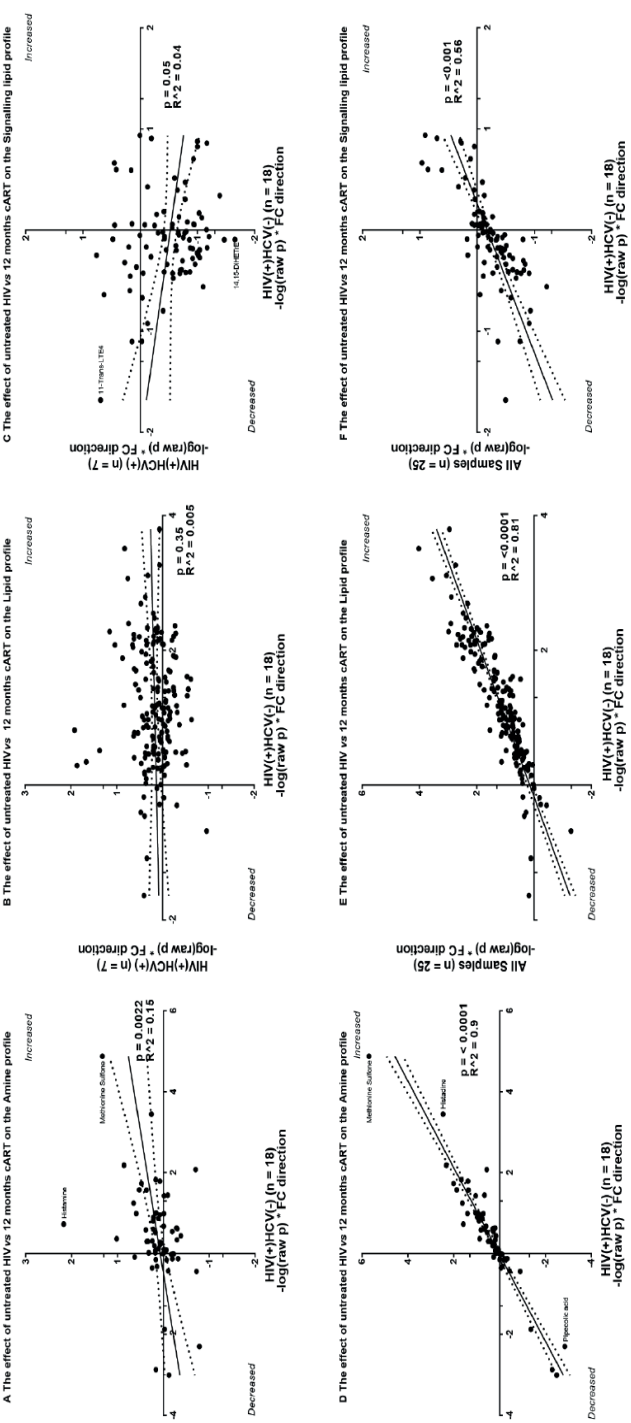


Figure S2 Directed p-value plots investigating metabolic changes in paired baseline (0 months) and 12 months cART samples (population B), through comparing the metabolic responses in different patient sub-populations. The directed p-value plots shown in A, B, and C for the three different metabolomics platforms compares the HIV(+)HCV(-) and HIV(+)HCV(+) sub-populations. The directed p-value plots shown in D, E, and F for the three different metabolomics platforms compares the HIV(+)HCV(-) and HIV(+) HCV(+/-)(All samples) sub-populations. A significant degree of correlation is found in D, E and F revealing that the HCV(+) populations has little influence in skewing the data and in actual effect somewhat dampening the observed effect of 12 months cART. A paired student T-test was used, and the direction of change was determined by the fold change direction. Pearson correlations were done per platform to investigate the degree of correlation with R^2 and p-values reported per graph, dashed lines represent 95% confidence intervals.

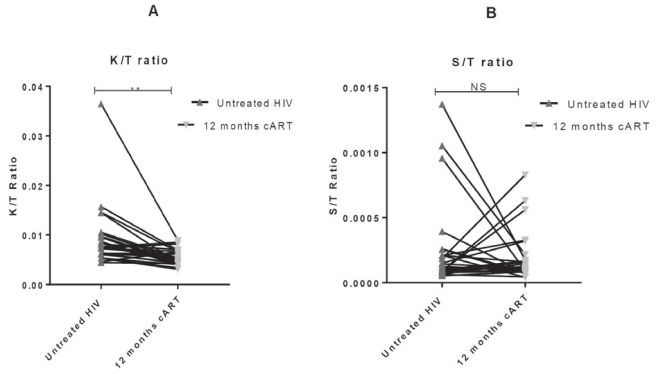


Figure S3: Tryptophan and Indoleamine 2,3-dioxygenase (IDO) activity during cART. (A) IDO activity decreased during 12 months of cART (population B). The kynurenine and tryptophan (K/T) ratio representative of IDO activity are plotted for the paired untreated HIV and 12 months cART samples. (B) The serotonin and tryptophan (S/T) ratio showed nonsignificant changes during the first 12months of cART in the same patients. Paired T-test with ** p< 0.01.

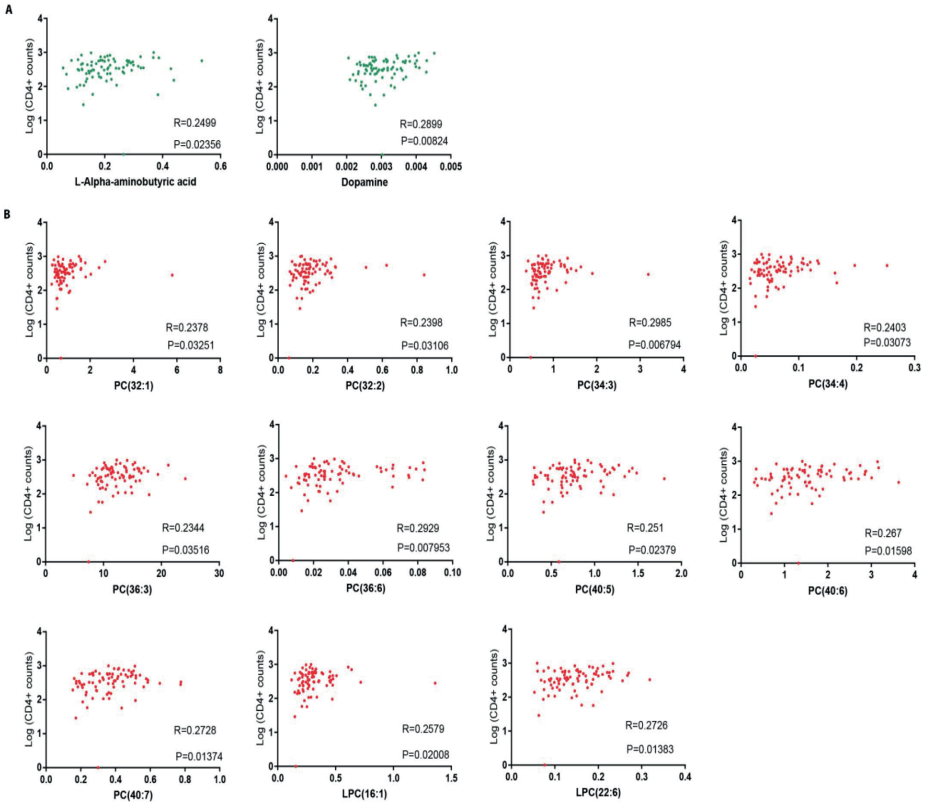


Figure S4: Significant correlations between the $CD4^+$ T-cell count and metabolite levels during 12 months of cART (population B). (A) Amine (green) metabolites correlated significantly ($p<0.05$) to $CD4^+$ T-cell counts. (B) Phospholipid (red) metabolites correlated significantly ($p<0.05$) to $CD4^+$ T-cell counts. All correlations were done using Spearman.

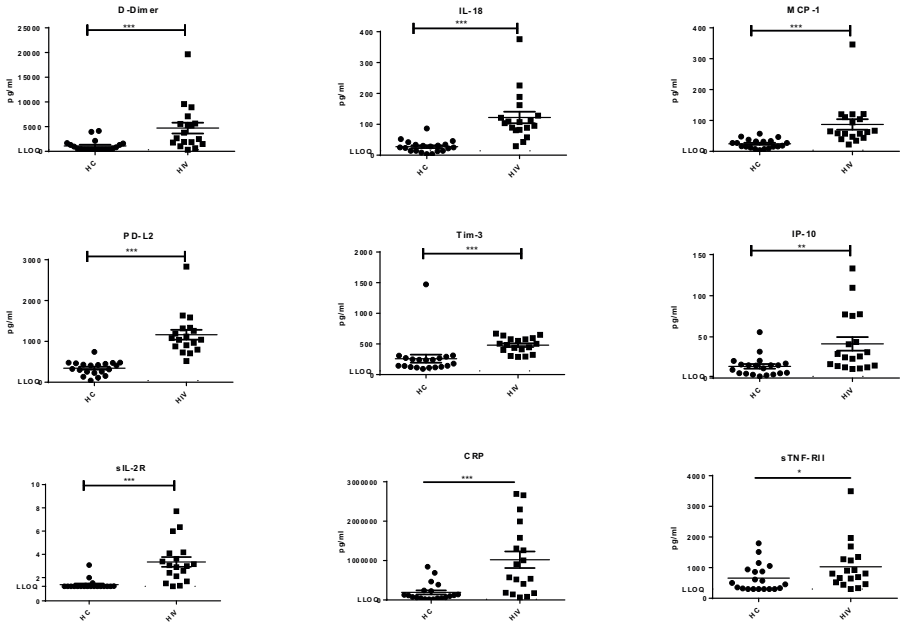


Figure S5: Plasma levels of the detected immunological markers in untreated HIV patients vs controls of population A. Increased levels of all markers were found during an untreated HIV infection. Unpaired T-test with p-values: * <0.05, ** <0.01, and *** <0.001

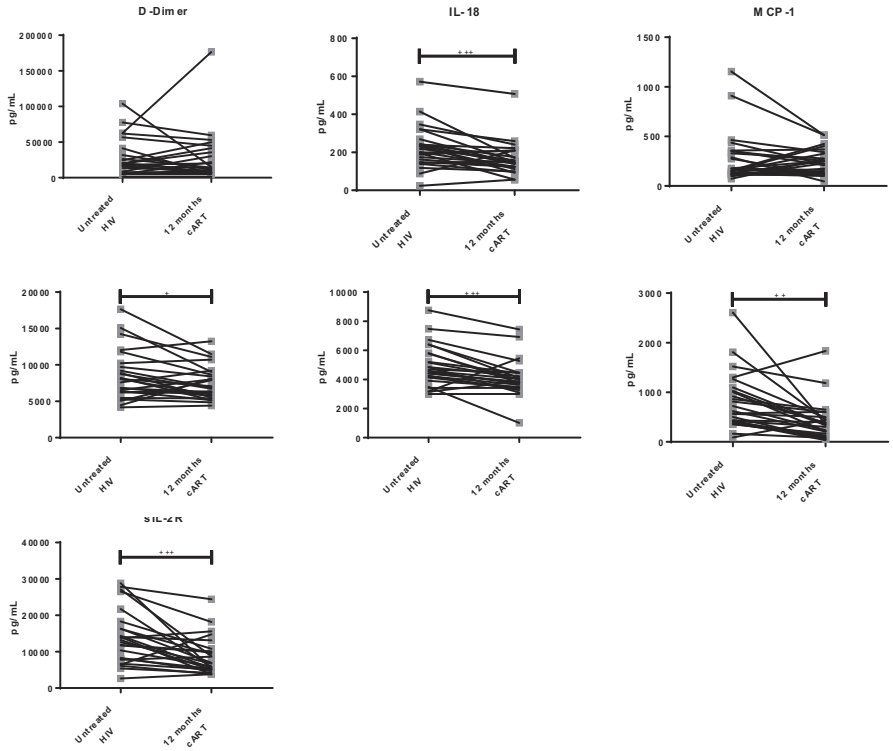


Figure S6: Plasma levels of the detected immunological markers in paired patient samples of population B. Decreasing levels of most markers were found during the first 12 months of cART. Paired T-test with p-values: + < 0.05, ++ < 0.01, +++ <0.001.

Table S1: Descriptive statistics of the biogenic amines comparing the untreated HIV patients to the controls in patient population A.

Metabolite	T-test	False discovery rate	Fold change	Controls	Untreated HIV
	p-value	q-value	(FC)	Mean ± SD[a]	Mean ± SD[a]
Glutathione	1.22E-11	3.88E-10	0.43017	0.12 ± 0.03	0.050 ± 0.01
O-Phosphoethanolamine	1.38E-11	3.88E-10	0.38745	3.71 ± 1.20	1.44 ± 0.53
Sarcosine	1.72E-10	3.21E-09	0.53698	0.13 ± 0.03	0.071 ± 0.02
Serotonin	4.05E-10	5.67E-09	0.24411	0.006 ± 0.00	0.002 ± 0.00
Taurine	6.14E-08	6.88E-07	0.50741	0.66 ± 0.26	0.33 ± 0.11
L-Tryptophan	1.59E-05	0.000149	0.76073	3.25 ± 0.51	2.47 ± 0.46
L-Alanine	0.000179	0.001432	0.76731	3.88 ± 0.79	2.98 ± 0.54
L-Histidine[b]	0.000396	0.002773	0.81706	0.037 ± 0.01	0.030 ± 0.01
L-Asparagine	0.000463	0.002882	0.77301	0.80 ± 0.18	0.62 ± 0.11
L-Alpha-aminobutyric acid	0.000848	0.004749	0.6884	0.27 ± 0.09	0.19 ± 0.04
L-Carnosine	0.000948	0.004826	0.63414	0.000 ± 0.00	0.000 ± 0.00
Ethanolamine[b]	0.002506	0.011693	0.85843	0.27 ± 0.03	0.23 ± 0.05
L-Leucine	0.002953	0.012719	0.7675	1.36 ± 0.36	1.04 ± 0.22
Gamma-L-glutamyl-L-alanine	0.004387	0.017546	0.73374	0.008 ± 0.00	0.006 ± 0.00
L-Methionine	0.005782	0.020692	0.75999	1.43 ± 0.45	1.09 ± 0.24
Putrescine	0.005912	0.020692	0.64703	0.001 ± 0.00	0.001 ± 0.00
L-Tyrosine	0.006596	0.021727	0.76809	9.06 ± 2.63	6.96 ± 1.61
L-Valine[b]	0.011206	0.034862	0.82117	7.46 ± 1.68	6.12 ± 1.20
Ornithine[c]	0.017497	0.051569	0.79523	1.63 ± 0.49	1.29 ± 0.37
S-Methylcysteine[c]	0.023788	0.066607	0.65039	0.13 ± 0.09	0.083 ± 0.03
L-Isoleucine[c]	0.025046	0.066789	0.78844	3.62 ± 1.16	2.86 ± 0.69
Gamma-Glutamylglutamine[b,c]	0.028906	0.07358	0.8311	0.007 ± 0.00	0.006 ± 0.00
L-Lysine[b,c]	0.035184	0.085666	0.83576	2.04 ± 0.55	1.71 ± 0.33

[a] SD, Standard Deviation; [b] Metabolites adhering to p-value<0.05 but not a FC of 30% (FC>1.3 or FC<0.7); [c] Metabolites having a FDR q-value>0.05

Table S2: Descriptive statistics of the lipid profiling comparing the untreated HIV patients to the controls in patient population A.

Metabolite	T-test	False discovery rate	Fold change	Controls	Untreated HIV
	p-value	q-value	(FC)	Mean ± SD[a]	Mean ± SD[a]
CE205[b]	0.0250	0.123	0.774	0.03 ± 0.01	0.02 ± 0.01
CE226[b]	0.0106	0.073	0.808	0.034 ± 0.01	0.027 ± 0.01
Cerd180_220[b]	0.0451	0.179	0.769	0.033 ± 0.01	0.025 ± 0.01
Cerd180_240[b]	0.0226	0.118	0.685	0.041 ± 0.02	0.028 ± 0.01
Cerd181_160[b]	0.0114	0.073	1.490	0.01 ± 0.00	0.02 ± 0.01
LPA-160	0.0003	0.002	2.005	0.09 ± 0.04	0.18 ± 0.13
LPA-161	0.0021	0.013	1.992	0.01 ± 0.01	0.03 ± 0.02
LPA-181	0.0017	0.011	1.847	0.10 ± 0.04	0.19 ± 0.15
LPA-182[b]	0.0256	0.083	1.515	0.56 ± 0.19	0.85 ± 0.57
LPA-203	0.0003	0.002	2.165	0.02 ± 0.01	0.04 ± 0.02
LPA-204	0.0000	0.000	2.047	0.15 ± 0.05	0.30 ± 0.16
LPC182[b]	0.0104	0.073	0.753	3.97 ± 1.30	2.99 ± 0.97
PC322[b]	0.0449	0.179	0.734	0.22 ± 0.09	0.16 ± 0.08
PC342[b,c]	0.0109	0.073	0.829	24.63 ± 5.54	20.42 ± 3.54
PC362[c]	0.0017	0.028	0.837	18.11 ± 2.80	15.16 ± 3.02
PC365[b]	0.0405	0.169	0.657	1.25 ± 0.73	0.82 ± 0.52
PC366[b]	0.0103	0.073	0.661	0.04 ± 0.02	0.02 ± 0.01
PC386	0.0003	0.012	0.702	5.56 ± 1.68	3.90 ± 1.17
PC404	0.0007	0.016	1.323	0.12 ± 0.04	0.16 ± 0.03
PC406	0.0014	0.026	0.712	1.64 ± 0.54	1.17 ± 0.45
PC407	0.0001	0.008	0.685	0.35 ± 0.10	0.24 ± 0.07
PC408	0.0001	0.008	0.653	0.08 ± 0.03	0.05 ± 0.02
PCO-341[b,c]	0.0081	0.072	0.833	0.40 ± 0.08	0.33 ± 0.08
PCO-342	0.0001	0.008	0.707	0.54 ± 0.13	0.38 ± 0.10
PCO-343	0.0008	0.016	0.690	0.52 ± 0.16	0.36 ± 0.13
PCO-363	0.0003	0.012	0.747	0.13 ± 0.03	0.10 ± 0.02
PCO-386[b]	0.0143	0.085	0.779	0.06 ± 0.04	0.05 ± 0.03
PCO-387[b,c]	0.0162	0.090	0.813	0.10 ± 0.02	0.08 ± 0.03
PE342[b]	0.0386	0.169	0.705	0.41 ± 0.20	0.29 ± 0.09
PE382	0.0045	0.050	0.703	3.13 ± 1.22	2.20 ± 0.70
PEO-385[b]	0.0200	0.108	0.777	0.67 ± 0.24	0.52 ± 0.18
PEO-387	0.0004	0.012	0.636	0.24 ± 0.09	0.16 ± 0.05
SMd181_180[b,c]	0.0406	0.169	0.839	1.00 ± 0.25	0.84 ± 0.22
SMd181_181[b]	0.0243	0.123	0.796	0.40 ± 0.12	0.32 ± 0.11
SMd181_201	0.0019	0.028	0.750	0.28 ± 0.08	0.21 ± 0.06
TG501[b]	0.0156	0.090	1.346	7.59 ± 4.97	10.22 ± 4.24
TG502[b]	0.0099	0.073	1.360	10.73 ± 5.05	14.59 ± 5.60

Table S2: Descriptive statistics of the lipid profiling comparing the untreated HIV patients to the controls in patient population A. (continued)

Metabolite	T-test	False discovery rate	Fold change	Controls	Untreated HIV
	p-value	q-value	(FC)	Mean ± SD[a]	Mean ± SD[a]
TG503[b]	0.0364	0.169	1.359	5.54 ± 2.88	7.52 ± 3.54
TG511[b]	0.0114	0.073	1.526	0.57 ± 0.51	0.86 ± 0.45
TG512[b]	0.0075	0.069	1.589	0.97 ± 0.64	1.55 ± 0.82
TG513[b]	0.0067	0.066	1.414	0.70 ± 0.28	0.99 ± 0.38
TG514[b]	0.0469	0.182	1.281	0.30 ± 0.12	0.38 ± 0.14
TG522[b]	0.0125	0.077	1.296	30.19 ± 7.26	39.13 ± 12.24
TG531[b]	0.0393	0.169	1.375	0.13 ± 0.08	0.18 ± 0.08
TG552[b]	0.0277	0.132	1.284	0.09 ± 0.03	0.12 ± 0.04
TG565	0.0036	0.043	1.593	2.00 ± 0.52	3.19 ± 1.54
TG566[b]	0.0052	0.054	1.573	2.73 ± 0.74	4.29 ± 1.96
TG585	0.0034	0.043	1.535	0.14 ± 0.04	0.22 ± 0.12
TG586	0.0020	0.028	1.514	0.28 ± 0.06	0.42 ± 0.19

[a] SD, Standard Deviation; [b] Metabolites having a FDR q-value>0.05; [c] Metabolites adhering to p< 0.05 but not a FC of 30% (FC>1.3 or FC<0.7).

Table S3: Descriptive statistics of the signalling lipid profiling. Comparing the untreated HIV patients to the controls in patient population A.

Metabolite	T-test	False discovery rate	Fold change	Controls	Untreated HIV
	p-value	q-value	(FC)	Mean ± SD[a]	Mean ± SD[a]
PGF2a[b,c]	0.01302	0.0509	1.15220	0.029 ± 0.004	0.033 ± 0.0065
PGE2[c]	0.03223	0.0904	2.10360	0.015 ± 0.005	0.028 ± 0.0255
12S-HHTrE[c]	0.03437	0.0943	1.48530	0.460 ± 1.018	0.684 ± 0.6429
5,6-DiHETrE	0.00000	0.0000	2.13540	0.006 ± 0.003	0.013 ± 0.0060
14,15-DiHETrE	0.00000	0.0000	1.55070	0.047 ± 0.009	0.073 ± 0.0212
11,12-DiHETrE	0.00000	0.0001	1.57940	0.036 ± 0.008	0.057 ± 0.0183
8,9-DiHETrE	0.00001	0.0002	1.64400	0.006 ± 0.002	0.010 ± 0.0033
5-HETE	0.00007	0.0008	1.86770	0.061 ± 0.024	0.114 ± 0.0457
15-HETE	0.00079	0.0060	1.61210	0.088 ± 0.040	0.143 ± 0.0593
15S-HETrE	0.00256	0.0138	1.68570	0.037 ± 0.023	0.062 ± 0.0319
12-HETE	0.00566	0.0252	2.26500	0.779 ± 0.298	1.765 ± 1.1447
12S-HEPE[c]	0.01717	0.0633	0.61502	0.263 ± 0.176	0.161 ± 0.1160
9/10-NO2-OA	0.00058	0.0047	0.49727	0.145 ± 0.117	0.072 ± 0.0477
11-HDoHE	0.00000	0.0000	0.25878	0.172 ± 0.074	0.045 ± 0.0180
2,3-dinor-8-iso-PGF2a	0.00000	0.0001	2.21740	0.086 ± 0.027	0.191 ± 0.0817
20-HETE	0.00223	0.0131	1.55120	0.071 ± 0.020	0.110 ± 0.0497
8,12-iPF2a-IV	0.00250	0.0138	0.80572	0.032 ± 0.006	0.025 ± 0.0087
8-iso-PGF2a	0.00533	0.0245	0.74017	0.133 ± 0.039	0.098 ± 0.0384
8-iso-PGE2[c]	0.04270	0.1102	2.61560	0.009 ± 0.006	0.024 ± 0.0188
SPHA-C180	0.00394	0.0195	1.66750	0.074 ± 0.023	0.123 ± 0.0825
S-1-P-161	0.00454	0.0217	0.74230	0.220 ± 0.069	0.163 ± 0.0450
S-1-P-182	0.00746	0.0321	0.77115	0.951 ± 0.250	0.734 ± 0.2526
S-1-P-181[c]	0.02076	0.0724	0.79819	2.914 ± 0.783	2.326 ± 0.7220

[a] SD, Standard Deviation; [b] Metabolites adhering to $p<0.05$ but not a FC of 30 % (FC>1.3 or FC<0.7); [c] Metabolites having a FDR q-value >0.05

Table S4: Descriptive statistics of the biogenic Amine and lipid profiling. Comparing the untreated HIV patients to their paired 12 months cART follow samples in patient population B.

Metabolite	Paired T-test	False discovery rate	T-stat[a]	Untreated HIV (baseline)	12 months cART
	p-value	q-value		Mean ± SD[b]	Mean ± SD[b]
Platform: Biogenic amines					
Methionine sulfone	2.0E-06	1E-04	6.14	0.008 ± 0.003	0.014 ± 0.007
L-Pipecolic acid	0.0014	3E-02	-3.59	0.046 ± 0.015	0.040 ± 0.016
DL-3-aminoisobutyric acid	0.0032	4E-02	-3.26	0.023 ± 0.011	0.018 ± 0.008
L-Histidine	0.0034	4E-02	3.23	0.022 ± 0.003	0.025 ± 0.004
L-Tryptophan	0.0047	5E-02	3.10	2.715 ± 0.891	3.243 ± 0.487
L-Kynurenine	0.0051	5E-02	-3.07	0.021 ± 0.006	0.017 ± 0.004
Platform: Lipid profiling					
CE181	0.0086	5E-02	2.86	0.039 ± 0.011	0.047 ± 0.009
LPC181	0.0079	5E-02	2.90	2.372 ± 0.681	2.751 ± 0.558
LPC183	0.0030	4E-02	3.30	0.054 ± 0.020	0.077 ± 0.029
LPC203	0.0065	5E-02	2.98	0.291 ± 0.103	0.369 ± 0.116
PC322[c]	0.0127	6E-02	2.69	0.149 ± 0.061	0.192 ± 0.068
PC341	0.0011	3E-02	3.71	12.278 ± 2.382	14.096 ± 2.540
PC343	0.0009	3E-02	3.80	0.658 ± 0.195	0.886 ± 0.269
PC344[c]	0.0115	6E-02	2.74	0.047 ± 0.031	0.070 ± 0.039
PC361	0.0003	2E-02	4.25	2.712 ± 0.891	3.663 ± 1.115
PC362	0.0012	3E-02	3.66	15.778 ± 3.018	18.441 ± 3.238
PC363	0.0018	4E-02	3.51	10.626 ± 2.977	13.234 ± 2.799
PC364[c]	0.0121	6E-02	2.71	11.041 ± 2.766	12.950 ± 2.812
PC382	0.0013	3E-02	3.64	0.322 ± 0.102	0.432 ± 0.124
PC383[c]	0.0165	7E-02	2.58	2.514 ± 1.250	3.699 ± 1.838
PC384[c]	0.0141	6E-02	2.65	7.179 ± 2.238	9.006 ± 2.590
PC385[c]	0.0141	6E-02	2.65	3.437 ± 1.338	4.544 ± 1.349
PC404[c]	0.0102	5E-02	2.79	0.169 ± 0.065	0.223 ± 0.083
PC405	0.0052	4E-02	3.07	0.605 ± 0.200	0.876 ± 0.292
PC406[c]	0.0226	8E-02	2.44	1.231 ± 0.415	1.821 ± 0.866
PC407	0.0083	5E-02	2.88	0.311 ± 0.115	0.418 ± 0.146
PC408	0.0033	4E-02	3.27	0.058 ± 0.022	0.077 ± 0.024
PCO-341	0.0039	4E-02	3.20	0.358 ± 0.068	0.398 ± 0.075
PCO-342	0.0001	2E-02	4.68	0.532 ± 0.123	0.669 ± 0.140
PCO-343	0.0033	4E-02	3.26	0.391 ± 0.129	0.466 ± 0.101
PCO-362	0.0026	4E-02	3.36	0.188 ± 0.033	0.212 ± 0.035
PCO-363	0.0022	4E-02	3.43	0.128 ± 0.028	0.152 ± 0.028
PCO-385	0.0048	4E-02	3.11	0.861 ± 0.162	0.986 ± 0.192
PCO-406	0.0062	5E-02	3.00	0.143 ± 0.021	0.169 ± 0.029

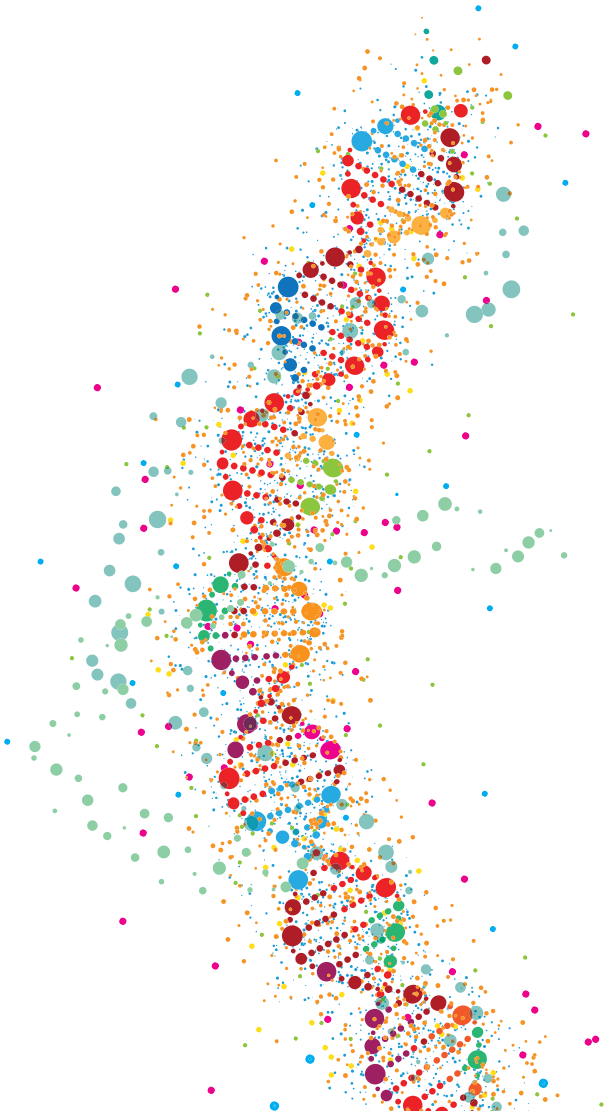
Table S4: Descriptive statistics of the biogenic Amine and lipid profiling. Comparing the untreated HIV patients to their paired 12 months cART follow samples in patient population B. (continued)

Metabolite	Paired T-test	False discovery rate	T-stat[a]	Untreated HIV (baseline)	12 months cART
	p-value	q-value		Mean ± SD[b]	Mean ± SD[b]
PE382	0.0010	3E-02	3.73	3.248 ± 0.995	4.343 ± 1.346
PE384[c]	0.0180	7E-02	2.54	0.203 ± 0.073	0.286 ± 0.146
PEO-365	0.0042	4E-02	3.16	0.300 ± 0.109	0.370 ± 0.144
PEO-385	0.0079	5E-02	2.90	0.460 ± 0.169	0.580 ± 0.243
SMd181/161[c]	0.0276	9E-02	2.35	1.033 ± 0.275	1.161 ± 0.248
SMd181/181[c]	0.0289	1E-01	2.32	0.706 ± 0.225	0.821 ± 0.244
SMd181/200	0.0033	4E-02	3.26	1.210 ± 0.274	1.470 ± 0.351
SMd181/201	0.0020	4E-02	3.47	0.403 ± 0.112	0.490 ± 0.142
SMd181/210[c]	0.0123	6E-02	2.71	0.367 ± 0.107	0.441 ± 0.128
SMd181/220	0.0067	5E-02	2.97	2.761 ± 0.647	3.173 ± 0.649
SMd181/221	0.0037	4E-02	3.21	1.866 ± 0.395	2.159 ± 0.393
SMd181/230	0.0084	5E-02	2.87	1.028 ± 0.250	1.176 ± 0.267
SMd181/231	0.0060	5E-02	3.01	0.758 ± 0.137	0.872 ± 0.170
SMd181/240[c]	0.0099	5E-02	2.80	1.827 ± 0.457	2.090 ± 0.431
SMd181/241[c]	0.0135	6E-02	2.67	6.005 ± 1.019	6.506 ± 1.023
SMd181/242	0.0084	5E-02	2.87	2.506 ± 0.575	2.751 ± 0.529
TG505[c]	0.0155	6E-02	2.61	0.178 ± 0.232	0.248 ± 0.223
TG525[c]	0.0257	9E-02	2.38	2.160 ± 2.715	2.727 ± 1.860
TG545[c]	0.0278	9E-02	2.34	7.446 ± 6.853	9.387 ± 5.391
TG546[c]	0.0223	8E-02	2.44	2.985 ± 3.473	3.687 ± 2.546
TG547[c]	0.0261	9E-02	2.37	0.688 ± 0.895	0.844 ± 0.650
TG563[c]	0.0192	7E-02	2.51	0.596 ± 0.582	0.756 ± 0.489

[a] For T-stat positive values: increased in 12 months cART vs untreated HIV. For T-stat negative values: decreased in 12 months cART vs untreated HIV; [b] SD, Standard Deviation; [c] Metabolites adhering to $p<0.05$ but not a FDR q-value of $q<0.05$

Chapter 8.

Summarizing discussion and future perspectives



INTRODUCTION

From an untreatable deadly illness HIV has become a chronic disease in less than 40 years, due to tremendous progress in treatment effectivity and tolerability. Nowadays, most patients are treated with only one combination tablet a day, whereas previously up to 16 pills were sometimes necessary. However, since HIV infection can still not be cured, HIV-infected patients need lifelong treatment and are at risk for treatment related adverse events. Some adverse events occur within the first months after the start of combination antiretroviral therapy (cART), such as gastro-intestinal complaints like nausea or abdominal pain, dizziness or headache and skin rashes. Other adverse events may only become evident years or even decades after the start of a cART regimen, such as nephrotoxicity, diabetes mellitus and bone mineral density loss. Apart from determining the genotype HLA-B*57:01 to predict a hypersensitivity reaction to abacavir, it is currently still not possible to predict which patient will suffer from adverse events due to cART. New biomarkers to predict adverse events would be valuable tools in the treatment of HIV.

Recent studies have given insight in the possible role of the *ITPA* genotype as a biomarker to predict adverse events during purine analogue therapy for hepatitis C (HCV). SNPs in the *ITPA* gene, leading to a decreased ITPase activity, were found to be protective against the development of hemolytic anemia during treatment with ribavirin.[1-7] Purine analogues are also used in the vast majority of cART regimens for HIV, these are abacavir and tenofovir. Thus, it can be hypothesized that the *ITPA* genotype is a potential genetic biomarker for adverse events associated with these drugs. However, the use of genetic biomarkers has disadvantages such as lack of sufficient evidence for consistent phenotype-genotype associations, significant overlap between genotype and influence of polygenic factors. These disadvantages may potentially be overcome by using a protein instead of a genetic biomarker. For the *ITPA* gene the translated protein is ITPase, an ubiquitous enzyme in humans. In this thesis, the influence of *ITPA* genotype and ITPase activity on the occurrence of adverse events during the use of purine analogue drugs for HIV and HCV was investigated and the correlation between *ITPA* genotype and ITPase activity was determined.

The current chapter summarizes and discusses the main findings of this thesis with emphasis on the difference between *ITPA* genotype and ITPase activity as biomarker and their role in the prediction of adverse events. Additionally, metabolomics after initiation of cART are discussed, as are recommendations for future research.

MAIN FINDINGS AND DISCUSSION

ITPA genotype versus ITPase activity

ITPA genotype was assumed to be directly associated with ITPase activity and the current literature has mainly focussed on SNPs in the *ITPA* gene as biomarkers for adverse events related to drugs targeting purine metabolism. However, *ITPA* genotype does not always directly correspond to ITPase activity. For instance, in HIV-infected patients, ITPase activity in erythrocytes is decreased compared to a HIV-negative control population, despite a similar allele frequency.[8] Therefore ITPase expression and activity were investigated in correlation with *ITPA* genotype for both HIV and HCV infected patients.

In **Chapter 2** it is described that in patients infected with HCV, *ITPA* genotype is not directly associated with ITPase activity. While the *ITPA* genotype wt/c.94C>A resulted in a decreased ITPase activity in all HCV-infected patients, the *ITPA* genotype wt/c.124+21A>C was associated with a wide variety of ITPase activities, ranging from 0.67 to as high as 5.47 mmol IMP/mmol Hb/hr (reference values measured in Caucasian populations are 4.0-10.0 mmol IMP/mmol Hb/hour).[9] Moreover, 38% of patients with this SNP had a normal ITPase activity. On the other hand, one of the patients with wt/wt *ITPA* genotype, presumed to have a normal ITPase activity, had a decreased activity. An explanation may be that in our study only part of the *ITPA* gene was sequenced to determine the prominent *ITPA* polymorphisms 94C>A and 124+21A>C only. However, more *ITPA* SNPs have become known[10-12] and we cannot exclude the possibility that the decreased ITPase activity in the patient with wt/wt genotype was due to another genetic variant in the *ITPA* gene. Because of the low prevalence of the other genetic variants, it was chosen not to determine these in the studies of this thesis.

In **Chapter 3,** presence of ITPase in leukocytes was determined using monoclonal anti-ITPase antibodies and the ITPase expression was quantitated by measuring median fluorescent intensity for the ITPase positive cell fraction. In all the leukocyte subtypes, except monocytes, of the HIV-infected patients ITPase presence was significantly decreased compared to the control population. Also, median fluorescent intensity was lower in HIV-infected patients in all cell types. No correlation of *ITPA* genotype with ITPase expression in leukocytes was observed.

Why ITPase expression and activity are decreased in erythrocytes and leukocytes of HIV-infected patients is yet unknown. A direct effect of nucleoside analogues on ITPase activity was excluded previously *in vitro*.[8] It is intriguing to think of HIV infection as a struggle for dominance over intracellular nucleotide pools. As such, decreased ITPase expression may be a defensive mechanism of the host cell. There is evidence that human leukocytes can

mount an offence against HIV-1 infection by decreasing their cytoplasmic deoxynucleotide triphosphate pools by increasing sterile alpha motif and histidine-apartic domain-containing protein 1 (SAMHD1) expression thus impeding DNA replication and repair, in turn inhibiting viral replication.[13] Earlier reports showed that HIV-infected T-lymphocytes were severely affected in both purine and pyrimidine nucleotide metabolism.[14-16] After exogenous mitogenic stimulation, intracellular adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP) pools declined dramatically rather than expanded as is required for proliferation.[16] In line with these observations, decreased ITPase activity may be the consequence of HIV targeting host cell nucleotide metabolism as well as another suicidal defense mechanism. By increasing non-canonical nucleotide pools, incorporation of these nucleotides into DNA and RNA is stimulated, leading to mutagenesis and, more importantly, programmed cell death, thus hindering HIV replication.[17]

A direct intracellular effect of HIV infection cannot be the only explanation for a decrease in ITPase activity. Also in erythrocytes and B-cells ITPase activity was found to be decreased, whereas these cells are not infected by HIV. An intracellular pathway activated by extracellular HIV particles may be hypothesized, because for B-cells, there is strong evidence that HIV binds to the CD21 receptor.[18] The influence of other factors secondary to HIV infection, such as chronic inflammation is unclear.

Taken together, decreased ITPase expression might be part of the host cell's response in defense to HIV-infection, or a consequence of HIV infection itself. On top of that there may be additional yet unidentified causes.

ITPase activity as a biomarker in predicting adverse events

There are several arguments in favor of preferring ITPase activity in erythrocytes as a biomarker over *ITPA* genotype to be used in clinical practice: 1) *ITPA* genotype is less strictly associated with ITPase activity than previously assumed,[8,19] 2) HIV-infection most probably influences ITPase activity,[8,20] 3) *ITPA* genotype did not influence ITPase expression in leukocytes,[20] (in both HIV-infected as well as control patients), and finally 4) unknown and rare SNPs not detected by routine screening for the most prevalent genetic variants may cause a decrease in ITPase activity. Therefore, the role of ITPase activity as biomarker for adverse events was evaluated and compared to *ITPA* genotype in the viral infections HCV and HIV.

Before ITPase can be used to predict adverse events during therapy with purine analogues, there are several issues that need to be addressed. First, the intra individual variability of the ITPase activity. In order to use a test as a biomarker, one has to know that the value found is reproducible, when measured at a different time. For ITPase the intra individual vari-

ability was found to be low (mean variation 15.2% within each subject)[21] and within- and between-day imprecision in ITPase activity measurement was 3.8% and 7.5% respectively.[10] Thus, under stable conditions, ITPase activity is a reproducible parameter, with a proper test available.

Further, the effect of other parameters (as age, sex and drugs) on ITPase activity is important to assess before using it as a biomarker. In several studies age had no effect on ITPase activity[21,22] nor had sex.[21-23] Multiple drugs were tested and found to have no inhibitory effect on ITPase activity: corticosteroids, infliximab, mesalazine, adalimumab,[21] azathioprine[22] and multiple nucleoside analogues (among which abacavir and TDF).[8] Cytarabine and gemcitabine were found to decrease ITPase activity in MOLT-3 cells after 18 hr of incubation,[8] but this effect is probably due to death of this cell population. Only ribavirin was thought to increase ITPase activity,[24] this topic will be discussed more in depth below.

Hepatitis C

In **Chapter 2** it was found that ITPase activity is a better biomarker than *ITPA* genotype to predict the development of anemia during ribavirin treatment for HCV infection. ITPase activity had a higher negative predicting value for the onset of anemia at both 4 weeks after the start of ribavirin and at the time of the lowest measured hemoglobin value during treatment. Therapy success rate, defined as sustained virological response (SVR), was not associated with either ITPase activity or *ITPA* genotype. During the time this study was done, standard treatment for HCV consisted of pegylated interferon alpha in combination with ribavirin. Nowadays, NS5A inhibitor, NS5B RNA-dependent RNA polymerase inhibitor and NS3/4A protease inhibitor combinations are the mainstay of HCV therapy, with cure rates above 95%. Ribavirin is now only used to reduce treatment duration in well-defined clinical scenarios or for patients with specific HCV genotypes or unfavourable patient characteristics (like cirrhosis) with known low rates of SVR.[25] By using ITPase activity as a predictor, adverse events may potentially be prevented during treatment with ribavirin in a more tailor-made treatment model for these hard to treat patients.

How and why low ITPase activity and or *ITPA* variants are associated with less anemia during treatment with ribavirin remains somewhat enigmatic and contradictory phenomena are observed. Previously it has been assumed that low ITPase activity would decrease ribavirin metabolite levels in erythrocytes, while quite obviously the opposite seems to be true.[26,27] This makes sense as ribavirin triphosphate makes an excellent substrate for ITPase.[28] Ribavirin was found to induce ITPase activity,[24] but there was no effect on ITP levels.[29] This also makes sense as ITP only accumulates to detectable levels in the erythrocytes when ITPase activity is completely lacking.[11,30] It is known that ribavirin depletes erythrocytes of ATP.[29,31] ATP is thought to play an important role in erythrocyte membrane stability. The effect of

ITPA genotypes on ribavirin-induced ATP depletion has been a focus of research, but the data are conflicting. An *in vitro* study showed a reduced ATP loss in erythrocytes from patients with ITPase activity lowering SNPs,[32] and an *in vivo* study showed a larger ATP decrease in individuals with these SNPs compared to individuals with wt/wt genotype.[29] The question rises whether ITP can function as an alternative energy source in erythrocytes as ITP was found not to be a substrate for ATPases keeping membrane stability in erythrocytes.[32] However, ITP was found to be a substitute for GTP as an energy source for adenylosuccinate synthethase (ADSS), which is the only known enzyme in erythrocytes to generate an adenine nucleotide from IMP.[32] Whereas in older literature it was described that ADSS is lacking in human erythrocytes,[33] a recent study showed traces of it with a deep proteomic analysis technique.[34] Thus potentially a decreased ITPase may lead to more availability of ITP (yet undetectable with present day techniques) in the erythrocytes, leading to generation of AMP from IMP via ADSS, restoring ATP depletion and protecting the erythrocyte from hemolysis.

In conclusion, although the mechanism is unclear, ITPase activity and *ITPA* genotype are biomarkers to predict hemolytic anemia during ribavirin therapy for HCV infection.

HIV

In HIV-infected patients, ITPase activity was also found to be a better predictor than *ITPA* genotype for adverse events during treatment with purine analogues, as described in **Chapter 4, 5 and 6**. An activity of ≥4 mmol IMP/mmol Hb/h was chosen as normal, being the lowest value within the 95% confidence interval for *ITPA* wild type carriers.[8,10] Tenofovir and abacavir are purine analogues frequently used in the backbone of the currently recommended cART regimens for HIV. Tenofovir disoproxil fumarate (TDF, referred to as tenofovir in this thesis) is a prodrug, which is phosphorylated inside the cells to tenofovir-diphosphate, which is the active metabolite and a non-canonical nucleotide 5'-triphosphate. Tenofovir-diphosphate will compete with the natural purine ATP for incorporation into the HIV DNA during its replication cycle. Abacavir is a guanosine analogue, and this prodrug is phosphorylated inside the cells to carbovir-triphosphate, the active metabolite competing with GTP. After incorporation of either tenofovir-diphosphate or carbovir-triphosphate into the growing HIV DNA-strand, further DNA synthesis is terminated because of the missing 3'-hydroxyl group in carbovir-triphosphate and tenofovir-diphosphate. Because didanosine, although being a guanosine nucleotide purine analogue, is no longer recommended in the treatment of HIV and no association was found between ITPase activity or *ITPA* genotype and adverse events during therapy with didanosine (**Chapter 4 and 6**), the focus in this discussion will be on abacavir and tenofovir.

Tenofovir

A decreased ITPase activity is associated with less occurrence of adverse events during the use of tenofovir in the cART regimen as is described in **Chapter 4**. In this retrospective study in 393 HIV-infected patients, all adverse events that led to stop of the cART regimen were analysed. Nephrotoxicity was less frequently found in patients with a decreased ITPase activity using tenofovir. This finding was confirmed in a retrospective cohort study (**Chapter 5**). Compared to the endpoints in **Chapter 4**, the endpoints in the latter study were more strictly defined. Nephrotoxicity was defined as >25% decrease in estimated glomerular filtration rate (eGFR) from the start of tenofovir use and/or the presence of ≥2 markers of proximal tubular dysfunction (PTD).[35] HIV-infected patients with (cases) and without (controls) tenofovir-associated nephrotoxicity were matched for age, gender and ethnicity. ITPase activity, *ITPA* genotype and the improvement of eGFR and PTD after tenofovir cessation were compared between both groups. 73% of the cases had a normal ITPase activity, compared to 50% of the controls (p=0.001). Remarkably, ITPase activity was also associated with recovery of renal function after stopping tenofovir. In patients with normal ITPase activity, the recovery of eGFR was significantly better than in the patients with decreased ITPase activity. *ITPA* genotype wt/wt was also associated with more renal adverse events compared to patients carrying 124+21A>C or 94C>A (**Chapter 4**). In **Chapter 5** the odds ratio for developing nephrotoxicity was not statistically significantly increased for patients with wt/wt genotype; 2.56 (95% CI 0.89-7.31). However, eGFR improvement after cessation of tenofovir was significantly better for wt/wt genotype.

It is an intriguing question what mechanism might be causing the protective effect of a decreased ITPase activity against nephrotoxicity. In tubular cells, tenofovir causes mitochondrial DNA (mtDNA) toxicity.[36-38] Imbalanced nucleotide pools may cause mtDNA depletion, potentially influenced by ITPase, thereby leading to mitochondrial dysfunction.[39,40] Hypothetically decreased ITPase activity could lead to an increase in ITP. Structurally, ITP and ATP are very similar and it is not unthinkable that ITP can substitute for ATP in reactions that are driven by ATP's high-energy phosphate esters.[41,42] Tenofovir is found to decrease the production of ATP in proximal tubular mitochondria.[43] Therefore, ITP may serve as an alternative high-energy donor during ATP depletion caused by tenofovir.

Tenofovir causes increased oxidative stress in mitochondria of renal tubular cells.[44] Oxidative stress leads to a relative increase of xanthine oxidase (XO) activity,[45] a source of free radicals.[46] With respect to ITPase activity and oxidative stress two scenarios can be envisioned. Fist, a relatively high, i.e. normal, activity drives the flux from ITP via IMP towards the production of hypoxanthine. Hypoxanthine is a substrate for XO, thereby a source of free radicals. Reasoning along this line, individuals with a normal ITPase activity may have higher levels of oxidative stress because of more availability XO substrates than individuals

with a low ITPase activity. The second scenario considers the assumed primary function of ITPase, which is the elimination of non-canonical purine nucleoside triphosphates. Oxidative stress leads to oxidized (damaged), thus non-canonical, nucleotides.[47] A high ITPase activity provides better protection against accumulation of rogue nucleotides than a low activity. Perhaps this is why individuals with normal ITPase activity experience more nephrotoxicity on tenofovir but also quickly recover once tenofovir is discontinued.

Abacavir

The results of the studies investigating ITPase activity as a potential biomarker to predict adverse events during abacavir use, are shown in **Chapter 4 and 6.** As opposed to regimens containing tenofovir, in regimens containing abacavir, significantly more adverse events occurred in patients with decreased ITPase activity: 61% versus 39% in patients with normal activity. This difference did not reach significance in the linear regression analysis, adjusting for confounding factors. On the other hand, metabolic adverse events (defined as dyslipidemia, use of lipid lowering therapy, diabetes mellitus or hypertension) occurred more frequently in patients with a normal ITPase activity, as is shown in **Chapter 6** (odds ratio 3.11, 95% CI 1.34-7.21, p = 0.008). Although wt/wt *ITPA* genotype was crudely associated with an increase in metabolic events, after adjusting for confounding factors this association disappeared. For cardiovascular diseases, no association with either ITPase or *ITPA* genotype could be demonstrated, probably due to the low incidence of these diseases.

As ribavirin-triphosphate proved to be a substrate for ITPase,[28] in **Chapter 4,** both carbovir-triphosphate and tenofovir-diphosphate were studied for their ability to function as a substrate for ITPase. Both proved not to be substrates for ITPase. The explanation for the association between ITPase activity and adverse events is thus not a simple effect of ITPase causing accumulation of toxic metabolites. Changes in cellular signal transduction may be hypothesized to play a role.

Vasorelaxation, smooth muscle proliferation and platelet aggregation are processes mediated by guanosine 3', 5'-cyclic monophosphate (cGMP). cGMP is synthesised from GTP by the enzyme soluble guanylate cyclase (sGC) and has a wide range of effects within human cells. One of these effects is vasodilatation by vasorelaxation in vascular smooth muscle[48-51] and expression of sGC in endothelial cells reduces hypertension.[52,53] cGMP is being studied as a target for pharmacological therapy in cardiovascular disease. Under hypoxic conditions, however, sGC was found to shift its substrate specificity from GTP to ITP, causing increased inosine 3', 5'-cyclic monophosphate (cIMP) instead of cGMP.[54,55] As opposed to cGMP, cIMP induces vasoconstriction and it was also found to be formed when intact porcine arteries were incubated with exogenous ITP.[54] In human platelets, formation of cGMP was decreased when they were incubated with carbovir-triphosphate.[56] Patients

using abacavir were found to have lower flow-mediated dilatation[57] (FMD; a non-invasive technique to assess endothelial function),[58] which significantly correlates with invasive testing of coronary endothelial function and coronary atherosclerosis.[59-61] In these studies ITP and cIMP were not measured, but since the effect of cIMP is vasoconstriction, it may be hypothesized that carbovir-triphosphate made sGC substrate specificity shift from GTP to ITP, forming cIMP. It would also explain a link between varying ITPase activity, potentially leading to more or less availability of ITP in cells, abacavir use and metabolic adverse events during treatment for HIV. To further test this theory, experiments need to be conducted to determine whether carbovir-triphosphate inhibits sGC, if sGC substrate specificity shifts to ITP instead of GTP by carbovir-triphosphate or if carbovir-triphosphate competes with GTP for sGC. The effect of a decreased ITPase activity on the substrate availability for sGC and the mechanism behind its protective effect against metabolic events also needs further evaluation.

Metabolic changes in HIV-infection and cART containing abacavir

The patients with a normal ITPase activity seem to have an increased risk for metabolic events during abacavir use compared to patients with decreased ITPase activity (**Chapter 6**). The life expectancy of HIV-infected patients increases, and nowadays patients aged 50-54 years make up the largest subpopulation of people living with HIV in the USA.[62] Consequently, cardiovascular diseases (CVD) and metabolic events will also increase in the HIV-infected population. It is still unclear to what extend HIV-infection causes an increase in CVD and metabolic risk and which part of the increased risk can be attributed to the use of cART. Use of some drugs in cART has been associated with an increased risk for CVD. Patients using protease inhibitors (PIs) were found to have an increased relative risk of CVD compared to patients using cART not containing PIs.[63] Abacavir use has been associated with an increased risk of CVD compared to regimens without abacavir,[63-65] however, other studies could not confirm this increased risk.[66,67] Lipid parameters were found to improve when cART containing abacavir was switched to a regimen containing tenofovir.[68] On the other hand, it is unknown whether HIV-infection itself induces changes that increase risk of CVD and whether, if so, if it can be undone by the use of cART. To further investigate the influence of cART on metabolic events, plasma metabolites of a HIV-infected population before start of cART were compared to a non-HIV infected control population (**Chapter 7**). Additionally, plasma metabolites of untreated HIV-infected patients were compared to those after 12 months of cART. The comparison of the biogenic amines, lipids and signalling lipid metabolites between untreated, active HIV-infection, and non-HIV infected control patients showed a profile of mainly decreased biogenic amines in untreated HIV-infection. The decreased concentrations of leucine, isoleucine and carnosine might point to an impaired muscle metabolism, whereas reduced levels of ornithine could suggest an affected ureum cycle and a potentially decreased ammonia clearance. After 12 months of abacavir

containing cART, only 3 amines (methionine sulfone, histidine and tryptophan) showed a significant increase versus the baseline measurement. The lipid profile showed an overall increasing trend after 12 months of cART, however the most striking finding was that 12 months of cART had no significant effect on the signalling lipids, the group including amongst others prostaglandins, thromboxanes and leukotrienes. The signalling lipids are metabolites that play an essential role in immunological crosstalk.[69] Thus although cART successfully suppresses HIV-RNA in blood and restores the number of $CD4^+$ lymphocytes, it seems to be unable to restore all the metabolic changes caused by the infection. This may be an explanation for the persistently increased occurrence of CVD in the HIV-infected population, next to a potential direct effect of cART on CVD.

FUTURE PERSPECTIVES

ITPase activity in patients with viral infectious diseases

The results of this thesis show that ITPase activity may serve as a biomarker to predict adverse events during therapy with purine analogues for the chronic infections HCV and HIV. More specifically, during use of the purine analogues ribavirin (HCV), tenofovir and abacavir (HIV), ITPase activity was found to be associated with specific adverse events: anemia and hemoglobin decline, nephrotoxicity and metabolic adverse events, respectively. As currently many non-HIV infected individuals start with tenofovir as pre-exposure prophylaxis (PrEP) against HIV, a prospective study determining ITPase activity in relation to the occurrence of nephrotoxicity is warranted in order to determine the value as biomarker for this adverse event in a non-HIV infected population. Nephrotoxicity can also be prevented by using tenofovir alafenamide (TAF) instead of tenofovir, as this was found to be equally effective in suppressing HIV in cART, but with significantly less nephrotoxicity. However, TAF has not yet been studied as PrEP. A model using tenofovir in one arm and TAF in the other can give information on both the efficacy of TAF as PrEP and the association between ITPase activity and *ITPA* genotype and adverse events during TAF or tenofovir use in the absence of HIV-infection.

Apart from nephrotoxicity, osteoporosis during tenofovir use is another interesting topic to investigate since ITPase plays a role in the formation of pyrophosphate, an inhibitor of bone mineralization.[70,71] In a preliminary study, bone mineral density (measured by Dual Energy X-ray Absorptiometry (DEXA) scanning) of 10 HIV-infected patients with decreased ITPase activity was compared to 22 patients with normal ITPase activity at 2 time points: just before switching off tenofovir-containing cART and 48 weeks thereafter. Bone mineral density of the lumbar spine was found to significantly increase after cessation of tenofovir in the patients with decreased ITPase activity, but not in the patients with

normal ITPase activity. In the hip, bone mineral density increased more in the patients with decreased ITPase activity than in the patients with normal activity, but these results did not reach significance. A larger, preferably prospective study, following HIV-infected patients on tenofovir containing cART compared to patients on cART not containing tenofovir, measuring bone mineral density through DEXA scanning after 24, 48 and 72 weeks and comparing outcome for patients with decreased versus normal ITPase activity, may give more insight in the association of ITPase activity and bone toxicity during tenofovir use in cART for HIV-infection.

While CVD and metabolic events are expected to increase in the HIV-infected population due to increasing age, more research, preferably large and prospective studies, should be done to further elucidate the role of ITPase activity and *ITPA* genotype in the occurrence of these events. Other adverse events that are known to occur during use of abacavir, such as gastro-intestinal adverse events, should be the scope of further research as well, as these are among the most frequent reasons for stopping this drug during cART.

An intriguing question is whether the headache and depression seen during triumeq use (a combination drug containing abacavir, dolutegravir and lamivudine) is associated with ITPase activity. The potential link between ITPase activity and these adverse events may be hypothesized by the findings that inosine has an inhibitory effect on Purkinje cerebral cells in rats,[72] inosine is an endogenous agonistic ligand for the benzodiazepine receptors in pig brains,[73] and pathogenic *ITPA* mutations cause an encephalopathy.[12,74]

Potentially a decreased ITPase activity may lead to an increase of the risk for malignancies. Studies have shown that both genetically engineered knock-out of ITPase expression in mice and naturally occurring genetic variants in the human population cause genetic instability. *ITPA* knock-out mice incorporated more deoxyinosine residues into embryonic DNA than controls.[75] In fibroblasts cultured from these embryos an increase in chromosomal aberrations and single-strand DNA breaks was observed.[75] In addition, in humans the SNP 94C>A in the *ITPA* gene was associated with a higher number of DNA mutations.[17] These observations are in line with the mutagenic properties of dITP[76] and the role of ITPase in the sanitation of the nucleotide pool.[77] The incidence of malignancies is increased in the HIV-infected patient population and certain malignancies are considered AIDS-defining cancers (Kaposi sarcoma, non-Hodgkin lymphoma and invasive cervical cancer).[78] Although cART has reduced the incidence of these AIDS-defining cancers,[79,80] still, the occurrence of cancer (both AIDS-defining and non-AIDS-defining) is higher compared to the general population.[79-82] Investigating the association between ITPase activity and the risk for malignancies is an important subject to investigate in the HIV-infected population.

In other virus infections purine analogues are also being used. For instance tenofovir is used to treat hepatitis B (HBV) infection, ribavirin is sometimes used as a treatment option for Respiratory syncytial virus (RSV), ganciclovir can be used to treat Cytomegalovirus (CMV) and acyclovir to treat Herpes simplex virus (HSV). If ITPase activity or *ITPA* genotype could be used as a biomarker prior to treatment for these infections, potentially adverse events like nephrotoxicity and hemolytic anemia can be prevented.

The findings in Chapter 7 on changes in metabolites during untreated HIV-infection, not fully restored by cART may function as a starting point for further unravelling the metabolic pathways affected by HIV. New insight in these pathways is important for cardiovascular risk management and prevention of malignancies.

ITPase activity in other populations

As was mentioned before, in other populations than patients with HIV- and HCV-infection, purine analogues are being used, like azathioprine in patients with multiple autoimmune diseases and 6-mercaptopurine in patients with acute lymphoblastic leukemia. The question rises in what patient population using ITPase activity as a biomarker for adverse events during purine analogue based therapy will be most rewarding. In the Asian populations the SNP 94C>A, and thus a decrease in ITPase activity, was the most prevalent compared to other populations.[83] Perhaps screening should therefore concentrate on these Asian populations. A larger trial that includes mainly Asian HIV-infected patients would be helpful to investigate the use of ITPase activity and *ITPA* genotype as a biomarker for predicting adverse events during cART in this population.

More studies are needed to obtain more insight in the role of non-canonical nucleotides (like inosine) in human cells. Besides from having deleterious effects, the other, potentially beneficial effects could gain insight in the basic cell metabolism, as well as have potential implications for future treatment of human disease. Inosine was found to be cardioprotective,[84] and is currently under investigation to slow down Parkinson's disease and amyotrophic lateral sclerosis (ALS),[85,86] via neuroprotective mechanisms of hypoxanthine. In the form of inosine pranobex, inosine is under research to alleviate influenza-like symptoms via immunomodulatory routes.[87] Further, purine metabolism is under the attention in the anticancer field where, among other things, inosine monophosphate dehydrogenase (IMPDH) inhibitors have shown anti-leukemic effects in a variety of acute myelogenous leukemia (AML) cell lines.[88] Under what conditions is inosine a substrate for sGC? What happens to intracellular nucleotide pools in cells under stress (like hypoxemia or due to certain drugs)? And what role does the variability in ITPase activity between patients play in these mechanisms? Could ITPase activity be used in predicting cardiovascular or neurological disease

in patients other than HIV-infected patients? These questions need to be answered in order to gain more insight in human metabolism, towards tailor-made therapy for every patient.

CONCLUSIONS OF THIS THESIS

In the leukocytes of HIV-infected patients the presence and expression of the enzyme ITPase is significantly decreased compared to non-HIV infected individuals. Further, the *ITPA* genotype c.124+21A>C is less strictly associated with ITPase activity than has previously been assumed. ITPase activity is a more accurate biomarker than *ITPA* genotype for predicting 1) anemia and hemoglobin-decrease during HCV therapy with ribavirin, 2) adverse events in general and nephrotoxicity during HIV therapy with tenofovir and 3) metabolic events during HIV therapy with abacavir, and an ITPase activity <4 mmol IMP/mmol Hb/hour was associated with a decrease in these adverse events. The situations in which a normal ITPase activity seemed to be more favourable than a decreased activity were limited to adverse events in general during HIV therapy with abacavir (however no longer significant logistic regression analysis) and the recovery of nephrotoxicity after tenofovir cessation. Metabolites changed by HIV-infection are not all fully restored to the levels of non-HIV infected patients in spite of successful suppression of the HIV-replication by cART.

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Chapter 9.

Nederlandse samenvatting.

HUMAAN IMMUNODEFICIËNTIEVIRUS

Het Humaan immunodeficiëntievirus (HIV) is een enkelstrengs ribonucleïnezuur (RNA)-virus dat specifiek $CD4^+$ T-helper lymfocyten, macrofagen en dendritische cellen infecteert. Na binding van een oppervlakte-eiwit op de HIV celmembraan met de CD4 receptor en één van de co-receptoren CCR5 of CXCR4 op deze gastheercellen, fuseren beide celmembranen met elkaar en komt de inhoud van de HIV-cel in het cytoplasma van de gastheercel. Het HIV gebruikt daar het enzym 'reverse transcriptase' en de nucleotiden van de gastheercel om van de enkele RNA-streng een dubbele deoxyribonucleïnezuur (DNA)-streng te maken. Dit DNA wordt via het HIV-enzym 'integrase' in het DNA van de gastheer ingebouwd. Via het nucleotide-metabolisme van de gastheer worden vervolgens van dit ingebouwde DNA nieuwe RNA-moleculen afgeschreven, welke na translatie nieuwe HIV-eiwitten leveren. Deze HIV-eiwitten worden in een deel van de celwand van de gastheercel weer verpakt tot nieuwe HIV-partikels, die nieuwe gastheercellen kunnen infecteren. Onbehandelde HIV-infectie leidt tot een vermindering van $CD4^+$ T-cel lymfocyten en uiteindelijk tot immuundeficiëntie, Acquired Immune Deficiency Syndrome; AIDS genoemd. Zonder behandeling leidt dit ziektebeeld tot het overlijden van de patiënt. De medicatie die wordt gebruikt om HIV te remmen, combinatie anti-retrovirale therapie (cART) genoemd, is in de laatste tientallen jaren steeds effectiever geworden. cART kan de virusreplicatie onderdrukken en zorgen voor (gedeeltelijk) herstel van het immuunsysteem. Er zijn meerdere stappen in de HIV-replicatie cyclus die een aangrijpingspunt vormen voor de medicatie. In een cART regime zitten altijd meerdere middelen uit verschillende klassen medicatie die op verschillende van deze aangrijpingspunten werken. De huidige richtlijnen schrijven allen een combinatie van minimaal 3 middelen voor, waarin twee NRTIs worden gecombineerd met of een niet-nucleoside reverse-transcriptase remmer (NNRTI), een protease remmer (PI) of een integrase remmer. Hoewel de huidige therapie de laatste jaren zeer sterk verbeterd is qua effectiviteit, kunnen we HIV nog altijd niet genezen en moet een patiënt de rest van zijn leven cART blijven gebruiken. Zoals bij vrijwel alle medicatie treden ook bij cART gebruik frequent bijwerkingen op, waarvan we, behoudens middels de genetische marker HLA-B*57:01 voor abacavir, niet kunnen voorspellen welke patiënt ze krijgt en hoe ernstig deze zullen zijn.

ITPASE

De replicatie van HIV en de werking van de NRTIs zijn afhankelijk van het nucleotide metabolisme van de gastheercel. De nucleotiden, adenine en guanine (purines) en cytosine en thymine (pyrimidines), zijn de bouwstenen van DNA. Deze nucleotiden zijn uitgebreid onderzocht en van hun nucleotide-5'-trifosfaat vorm is bekend dat ze betrokken zijn bij

de energielevering voor verschillende processen in de menselijke cel, signalen doorgeven binnen de cellen, betrokken zijn bij de vorming van polysachariden en onderdelen zijn van verschillende co-enzymen die belangrijk zijn voor oxidatie-reductie reacties. Naast deze 4 nucleotiden, zijn er nog andere nucleobasen, zoals xanthine en hypoxanthine, waarvan de laatste de base is om de nucleoside inosine te vormen. Deze nucleobasen worden gevormd binnen de metabole paden van het purine nucleotide mechanisme, maar ook bij oxidatieve stress en deaminatie. De rol van xanthine en hypoxanthine, ook wel 'non-canonische' nucleosiden genoemd, is minder uitgebreid onderzocht dan van de 'canonische' nucleosiden. Wel is bekend dat ze zowel gunstige als schadelijke effecten kunnen hebben. Inosine trifosfaat pyrofosfohydratase (ITPase) is een enzym dat inosine-5'-trifosfaat (ITP) defosforyleert naar inosine-5'-monofosfaat (IMP). Dit enzym wordt gecodeerd door het gen *ITPA*. Verschillende single nucleotide polymorfismen (SNPs) zijn bekend binnen dit gen, waarvan meerdere SNPs leiden tot een verminderde activiteit van het enzym ITPase. Zo leidt homozygoot dragerschap voor SNP 94C>A tot nagenoeg volledige ITPase deficientie, terwijl heterozygote dragers van deze SNP nog 23% restactiviteit hebben. De SNP 124+21A>C leidt in homozygote dragers tot 30% en in heterozygote dragers tot 60% ITPase restactiviteit. Deze verschillende SNPs komen wereldwijd in wisselende frequenties voor bij verschillende populaties. Er is uitgebreid onderzoek gedaan naar de invloed van deze SNPs op bijwerkingen van purine analogen zoals azathioprine dat gebruikt wordt in de behandeling van inflammatoire darmziekten, mercaptopurine voor acute lymfoblastaire leukemie en ribavirine voor hepatitis C (HCV). Voor de eerstgenoemde middelen zijn de uitkomsten van de onderzoeken niet eenduidig. Voor ribavirine is echter aangetoond dat dragerschap van een SNP in het *ITPA* gen, leidend tot een vermindering van de activiteit van ITPase, beschermt tegen de bijwerking hemolytische anemie. In cART voor HIV worden purine analogen gebruikt als nucleoside/nucleotide reverse-transciptase remmers (NRTIs) zoals abacavir, tenofovir en didanosine.

Dit proefschrift beschrijft de resultaten van onderzoek naar het enzym ITPase als mogelijke biomarker voor het voorspellen van bijwerkingen tijdens de behandeling met ribavirine voor HCV en cART voor HIV en wordt hierin vergeleken met het *ITPA* gen.

ITPASE ALS BIOMARKER VOOR BIJWERKINGEN

In **hoofdstuk 2** wordt beschreven dat de ITPase activiteit een betere biomarker is dan het *ITPA* genotype voor het voorspellen van anemie en hemoglobine daling tijdens de behandeling van HCV met ribavirine. In alle in dit proefschrift beschreven onderzoeken wordt een verlaagde ITPase activiteit gedefinieerd als <4 mmol IMP vorming per mmol hemoglobine per uur (<4 mmol IMP/mmol Hb/uur), omdat dit de laagste waarde is van het 95%

betrouwbaarheidsinterval van de waarde van ITPase activiteit bij mensen met een *ITPA* genotype zonder SNPs (die geacht worden een normale ITPase activiteit te hebben). Uit deze studie, die 106 patiënten bevat, blijkt dat een verlaagde ITPase activiteit geassocieerd is met een minder laag hemoglobine, minder grote hemoglobine daling en minder frequent voor komen van anemie (bloedarmoede). Ook patiënten met een van beide hierboven genoemde SNPs in het *ITPA* gen blijken een significant minder laag hemoglobine en een mindere hemoglobine daling te hebben, maar het voorkomen van anemie is alleen verminderd in de patiënten met de SNP 94C>A vergeleken met patiënten zonder SNP in hun genotype (wildtype; wt/wt). Een mogelijke verklaring hiervoor is dat de SNP 124+21A>C in het *ITPA* gen minder strikt geassocieerd is met een verminderde ITPase activiteit dan eerder werd aangenomen. De patiënten met deze SNP hebben namelijk ITPase activiteiten variërend van sterk verlaagde tot normale waarden; 38% heeft een normale ITPase activiteit. Tot slot blijkt dat zowel de ITPase activiteit als het *ITPA* genotype geen invloed hebben op de effectiviteit van de HCV behandeling.

In **hoofdstuk 3** worden de resultaten beschreven van het onderzoek naar de aanwezigheid van het enzym ITPase in witte bloedcellen (leukocyten) van 59 HIV-patiënten vergeleken met een controle groep van 50 niet-HIV geïnfecteerde mensen. Het enzym ITPase blijkt in alle leukocyten subsets van HIV-patiënten, behalve de monocyten, minder vaak aanwezig te zijn dan in de leukocyten subsets van de controle groep. De cellen die wel ITPase bevatten, hebben bij de HIV-patiënten een minder sterke mate van expressie vergeleken met de cellen van de individuen in de controle groep. Deze verlaging van de aanwezigheid van ITPase is niet gerelateerd aan het *ITPA* genotype, want de verdeling van de genotypes is in HIV-patiënten hetzelfde als in de niet-HIV geïnfecteerde mensen. De resultaten van dit onderzoek sluiten aan bij eerder onderzoek, waarin ook in rode bloedcellen (erythrocyten) van HIV-patiënten een verlaagde ITPase activiteit werd gevonden ten opzichte van een controle populatie.

Naar aanleiding van bovenstaande bevindingen (1] ITPase activiteit lijkt een betere voorspeller te zijn van bijwerkingen tijdens therapie met een purine analoog voor HCV en 2] de ITPase activiteit is verlaagd in zowel erythrocyten als leukocyten van HIV-patiënten), werden de studies gedaan die worden beschreven in **hoofdstukken 4, 5 en 6**. In analogie van HCV, blijkt voor het voorspellen van bijwerkingen tijdens behandeling met de purine analogen abacavir en tenofovir in cART voor HIV de ITPase activiteit ook een betere maat dan het *ITPA* genotype. Didanosine is ook een purine analoog, maar omdat bij het gebruik hiervan geen relatie met ITPase activiteit en *ITPA* genotype is gevonden en dit middel tegenwoordig bijna niet meer gebruikt wordt, worden deze resultaten hier niet apart besproken.

Tenofovir

In **hoofdstuk 4** worden de resultaten beschreven van een retrospectieve studie in een populatie van 393 HIV-geïnfecteerde patiënten die behandeld werden met cART. De bijwerkingen worden gegroepeerd in de categorieën maag/darmklachten, neurologische klachten, nefrotoxiciteit, huidafwijkingen en leverproblemen. Bijwerkingen ten tijde van het gebruik van de verschillende cART regimes worden vergeleken tussen patiënten met een verlaagde en patiënten met een normale ITPase activiteit. Ook wordt deze vergelijking gemaakt tussen patiënten met een of meerdere van de SNPs c.94C>A en c.124+21A>C in het *ITPA* gen en de patiënten zonder SNPs in dit gen (wt/wt). De patiënten met een verlaagde ITPase activiteit hebben minder bijwerkingen dan de patiënten met een normale ITPase activiteit tijdens de behandeling met een cART regime dat tenofovir bevat. Dit geldt ook voor patiënten met SNPs in het *ITPA* gen (c.94C>A en/of c.124+21A>C) vergeleken met patiënten met het wildtype (wt/wt) *ITPA* genotype. Maar als gekeken wordt naar afzonderlijke bijwerkingen, dan is een verlaagde ITPase activiteit alleen geassocieerd met minder nierschade (nefrotoxiciteit) tijdens behandeling met tenofovir en kan deze associatie niet worden aangetoond voor het *ITPA* genotype. In het vervolgonderzoek (beschreven in **hoofdstuk 5**), vergelijken we HIV-patiënten die tenofovir gebruikten met nefrotoxiciteit tijdens therapie (26 patiënten) met 55 gematchte patiënten zonder nefrotoxiciteit. In deze studie worden de resultaten uit **hoofdstuk 4** bevestigd. Patiënten met nefrotoxiciteit hebben significant vaker een normale ITPase activiteit dan patiënten zonder nefrotoxiciteit. Dit verschil wordt niet gevonden als de verschillende *ITPA* genotypes met elkaar worden vergeleken. Wel is het hebben van een normale ITPase activiteit geassocieerd met een beter herstel van de nierfunctie na het staken van tenofovir.

Abacavir

Voor abacavir zijn de resultaten, beschreven in **hoofdstuk 4 en 6** minder eenduidig dan voor tenofovir. De bijwerkingen waarvoor een verminderde ITPase activiteit bij tenofovir beschermend lijkt te zijn (maag/darmklachten, neurologische klachten, nefrotoxiciteit, huidafwijkingen en leverproblemen), komen juist vaker voor bij de patiënten met een verminderde ITPase activiteit tijdens therapie met abacavir. Echter na correctie voor de factoren: duur van cART, duur van purine therapie en duur van het huidige cART regime, is dit verschil niet significant meer. Het *ITPA* genotype is niet geassocieerd met deze bijwerkingen tijdens therapie met abacavir.

In **hoofdstuk 6** blijkt dat het hebben van een verlaagde ITPase activiteit ook voor HIV-patiënten die abacavir gebruiken op een andere wijze gunstiger is, omdat zij minder vaak een metabole bijwerking (diabetes mellitus, hypertensie en dyslipidemie) blijken te hebben dan de patiënten met een normale ITPase activiteit. Hoewel ook in *ITPA* genotypes die een SNP bevatten minder metabole bijwerkingen lijken voor te komen, is deze associatie niet

langer significant na correctie voor leeftijd, body mass index (BMI), cumulatief gebruik van cART en het gebruik van een PI of NNRTI. Het eindpunt hart- en vaatziekten (cardiovasculaire ziekten) is in dit onderzoek niet geassocieerd met ITPase activiteit of *ITPA* genotype, waarschijnlijk door het lage aantal gevallen dat voorkwam in de onderzochte populatie.

MECHANISME

De ITPase activiteit is geassocieerd met bijwerkingen ten tijde van therapie met tenofovir (algemene bijwerkingen en nefrotoxiciteit) en abacavir (metabole bijwerkingen) voor HIV en zou daarom mogelijk in de toekomst in kunnen worden gezet als biomarker voor het voorspellen van bijwerkingen. De ITPase activiteit lijkt in de studies beschreven in dit proefschrift een betere biomarker te zijn dan het *ITPA* genotype. Een voor de hand liggende verklaring voor de gevonden associatie zou kunnen zijn dat de actieve metabolieten van tenofovir en abacavir een substraat zijn voor het ITPase enzym en dat zo de activiteit van ITPase de vorming van toxische metabolieten beïnvloedt. Dit is echter getest en beide blijken geen direct substraat te zijn, waardoor deze hypothese moet worden verworpen (**hoofdstuk 4**). In de discussie (**hoofdstuk 8**) van dit proefschrift worden enkele hypothesen besproken over de mogelijke mechanistische verklaring achter de gevonden associaties. Mogelijk spelen verschuivingen in intracellulaire nucleotide voorraden of het ontstaan van zuurstofradicalen een rol, maar ook een veranderde cellulaire signaal transductie waarin mogelijk ITP een substraat is voor cyclisch guanosine-3',5'-monofosfaat (cGMP) behoort theoretisch tot de mogelijkheden. Duidelijk is dat er nog veel aanvullend onderzoek nodig is om het mechanisme achter de gevonden associaties in zijn geheel te verklaren.

VERANDERINGEN VEROORZAAKT DOOR VIRUS OF MEDICATIE?

Een normale ITPase activiteit tijdens het gebruik van abacavir voor HIV-infectie zou dus ongunstig kunnen zijn voor onder andere het ontwikkelen van risicofactoren voor cardiovasculaire ziekten. Voor HIV-patiënten is gebleken dat het risico op cardiovasculaire ziekten verhoogd is, maar onbekend is in hoeverre dit risico wordt veroorzaakt door de infectie zelf of door de cART. Om meer inzicht te krijgen in de metabole veranderingen tijdens een actieve, onbehandelde HIV-infectie, worden in **hoofdstuk 7** metabolieten in het plasma van 18 HIV-patiënten vergeleken met dat van 23 niet-HIV geïnfecteerde personen. In de onbehandelde HIV-patiënten worden meerdere verschillen gezien ten opzichte van de controle groep in de biogene amines, waarvan alle amines die verschilden, verlaagd zijn bij HIV-patiënten. Ook het lipidenprofiel verschilt tussen beide groepen met een toename van meervoudig onverzadigde triglyceride soorten in HIV, maar een afname van fosfolipiden.

Binnen de groep signaallipiden, met name van belang voor het doorgeven van signalen tussen immuuncellen, is een groot deel van de lipiden verhoogd, waaronder de lipiden afkomstig van de vorming van oxidatieve zuurstofradicalen. Concluderend bestaan er grote verschillen tussen HIV-patiënten en niet-HIV geïnfecteerde personen in zowel biogene amines als lipiden en signaallipiden. De resultaten van dit onderzoek kunnen bijdragen aan toekomstig onderzoek dat noodzakelijk is voor het beantwoorden van meerdere vragen, zoals waarom HIV-patiënten een verhoogd cardiovasculair risico hebben, of waarom er meer maligniteiten voorkomen bij HIV-patiënten. De meest opvallende bevinding van het onderzoek dat wordt beschreven in **hoofdstuk 7** is dat 12 maanden behandeling met abacavir bevattende cART in HIV-geïnfecteerde patiënten met succesvolle onderdrukking van de virale replicatie toch niet leidt tot herstel van de gevonden verschillen tussen onbehandelde HIV-patiënten en de controle populatie. Slechts enkele verlaagde amines nemen significant toe na 12 maanden cART en alle signaallipiden blijven verlaagd, ondanks de therapie. De overige lipiden laten wel veranderingen zien en blijken over het algemeen toe te nemen tijdens therapie. Samenvattend betekent dit dat de metabole veranderingen die ontstaan tijdens actieve HIV-infectie, niet compleet ongedaan worden gemaakt met een jaar lang succesvolle abacavir bevattende therapie tegen HIV.

CONCLUSIES

In **hoofdstuk 8** worden alle resultaten samengevat en wordt de balans opgemaakt tussen ITPase activiteit en *ITPA* genotype als biomarker voor bijwerkingen tijdens de behandeling met de purine analogen ribavirine voor hepatitis C en abacavir en tenofovir voor HIV. Ook de mogelijkheden voor toekomstig onderzoek worden hier besproken. De belangrijkste conclusies van dit proefschrift zijn:

1) In de leukocyten van HIV-patiënten is het enzym ITPase minder vaak aanwezig en is de expressie minder sterk dan in de leukocyten van niet-HIV geïnfecteerde mensen.
2) Het voorkomen van de SNP c.124+21A>C is minder sterk geassocieerd met een verminderde ITPase activiteit dan voorheen werd aangenomen.
3) ITPase activiteit is een betere biomarker dan *ITPA* genotype voor het voorspellen van:
    a) anemie en hemoglobine-daling ten tijde van behandeling met ribavirine voor hepatitis C,
    b) bijwerkingen in het algemeen en nefrotoxiciteit in het bijzonder ten tijde van behandeling met tenofovir voor HIV,
    c) metabole bijwerkingen ten tijde van behandeling met abacavir voor HIV.
4) Bij alle bovengenoemde associaties is een verlaagde ITPase activiteit (<4 mmol IMP/mmol Hb/uur) gunstiger dan een normale ITPase activiteit.
5) Een verlaagde ITPase activiteit is echter mogelijk geassocieerd met

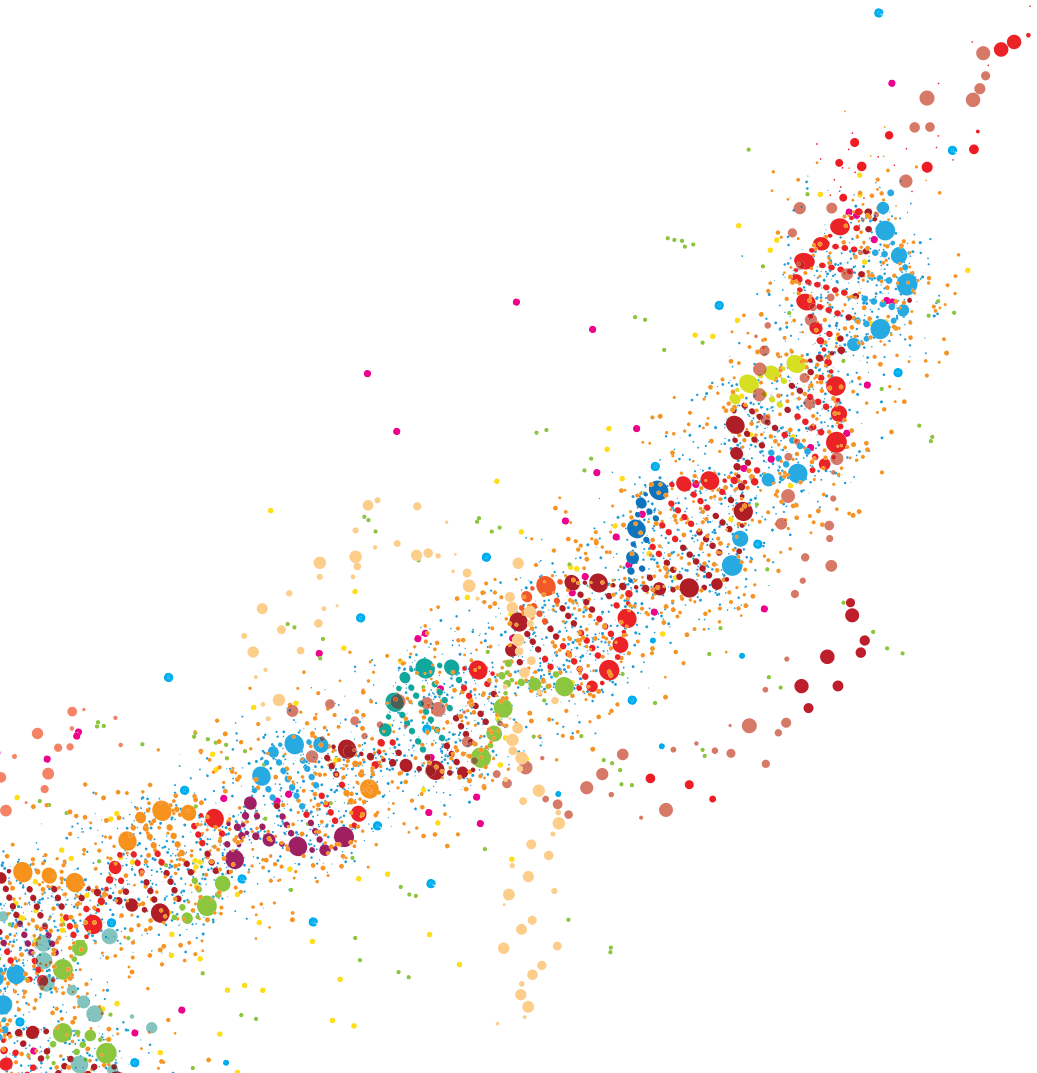
a) een slechter herstel van nefrotoxiciteit na het staken van tenofovir voor HIV,
b) het vaker voorkomen van algemene bijwerkingen tijdens gebruik van abacavir voor HIV.

6) De verandering in metabolieten bij patiënten met onbehandelde HIV-infectie, wordt niet volledig hersteld door een jaar succesvolle onderdrukking van de virale replicatie van HIV door gebruik van abacavir bevattende cART.

Toekomstig onderzoek zal uit moeten wijzen of ITPase ook bij andere indicaties voor tenofovir geassocieerd is met bijwerkingen en of het ook met andere bijwerkingen dan anemie, nefrotoxiciteit en metabole effecten geassocieerd is tijdens therapie met purine analogen. Verder zal onderzocht moeten worden in welke populaties de biomarker het best inzetbaar zal zijn. Ook algemeen onderzoek naar ITPase en de non-canonische nucleotiden is nodig om zowel hun gunstige als schadelijke effecten beter in kaart te brengen. Hopelijk zal dit leiden tot 'therapie op maat' voor elke patiënt.

Chapter 10

List of publications
PhD portfolio
Curriculum vitae
Dankwoord



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- **Peltenburg N.C.***, Wijting I.E.A.*, Rokx C., Bakker, J.A., Rijnders B.J.A., Bierau J. and Verbon A. Inosine 5'-triphosphatase activity is associated with TDF-associated nephrotoxicity in HIV. *Submitted. * Joint first authorship.*
- **Peltenburg N.C.***, Schoeman J.C.*, Hou J., Mora F., Harms A.C., Lowe S.H., Bierau J., Bakker J.A., Verbon A., Hankemeier T. and Boonstra A. Persistent metabolic changes in HIV-infected patients during the first year of combination antiretroviral therapy. *Scientific Reports* 2018. Nov 16; 8(1): 16947. PubMed PMID: 30446683. * *Joint first authorship.*
- **Peltenburg N.C.**, Bierau J., Schippers J.A., Lowe S.H., Paulussen A.D.C., Van den Bosch B.J.C., Leers M.P.G., Andrinopoulou E.R., Bakker J.A. and Verbon A. Metabolic events in HIV-infected patients using abacavir are associated with erythrocyte inosine triphosphatase activity. *Journal of Antimicrobial Chemotherapy* 2019 Jan 1; 74(1): 157-164. PubMed PMID: 30304447
- **Peltenburg N.C.**, Bierau J., Bakker J.A., Schippers J.A., Lowe S.H. Paulussen A.D.C., Van den Bosch B.J.C., Leers M.P.G., Hansen B.E. and Verbon A. Erythrocyte inosine triphosphatase activity: a potential biomarker for adverse events during combination antiretroviral therapy for HIV. *PLoS One* 2018. Jan 12; 13(1): e0191069. PubMed PMID: 29329318
- **Peltenburg N.C.**, Leers M.P.G., Bakker J.A., Lowe S.H., Vroemen W.H.M., Paulussen A.D.C., Van den Bosch B.J.C, Bierau J. and Verbon A. Inosine triphosphate pyrophosphohydrolase expression: decreased in leukocytes of HIV-infected patients using combination antiretroviral therapy. *Journal of Acquired Immune Deficiency Syndromes* 2016 Dec 1; 73(4): 390-395. PubMed PMID: 27792682
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PHD PORTFOLIO

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Erasmus MC Departments:	Internal Medicine and Medical Microbiology and Infectious Diseases
Promotor:	Prof. Dr. Annelies Verbon
Copromotors:	Dr. Jörgen Bierau and Dr. Jaap A. Bakker

Presentations

2014 Oral presentation, Spring congress Dutch Association for Clinical chemistry and laboratory medicine, Veldhoven. Winner category 'clinical'

2015 Oral presentation, Science Days Internal medicine, Antwerp, Belgium

2016 Poster presentation, Spring congress Dutch Association for Clinical chemistry and laboratory medicine, Veldhoven, The Netherlands

2016 2 Poster presentations, 26th ECCMID, Amsterdam, The Netherlands

2016 Poster presentation, International congress on drug therapy in HIV infection, Glasgow, United Kingdom

2016 Oral presentation, MMIZ research meeting, Rotterdam, The Netherlands

2016 Poster presentation, Science Days Internal medicine, Antwerp, Belgium

2017 Oral presentation, Midsummer meeting NVHB, Utrecht, The Netherlands

2017 Oral presentation, MMIZ research meeting, Rotterdam, The Netherlands

2019 Oral poster presentation, 29th ECCMID, Amsterdam, The Netherlands

(Inter)national conferences and meetings

2011 2x MINC symposium Mycology and immunosuppressive therapy and infections, Maastricht, The Netherlands

2012 2nd Euregional Maastricht symposium on Immune Compromised Travelers, Maastricht, The Netherlands

2012 NIV dagen, Maastricht, The Netherlands

2012 Symposium Infectious diseases, AMC, Amsterdam, The Netherlands

2012 Tropical medicine meeting, AMC, Amsterdam, The Netherlands

2013 2x MINC symposium Gastro-intestinal infections and Immunogenetics, Maastricht, The Netherlands

2014 MINC symposium Infections in nursing homes, Maastricht, The Netherlands

2014 3th Euregional Maastricht sumposium on Immune Compromised Travelers, Maastricht, The Netherlands

2014 HIV drug therapy congress, Glasgow, United Kingdom

2015 11th Workshop on HIV and hepatitis co-infection, London, United Kingdom

2015 Update and future challenges in the nationals and global fight against HCV, HBV, HIV, TB and Tropical diseases, Arnhem, The Netherlands
2016 26th European Congress on Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, The Netherlands
2016 International congress on drug therapy in HIV infection, Glasgow, United Kingdom
2017 NIV dagen, Maastricht, The Netherlands
2017 Midsummer meeting NVHB, Utrecht, The Netherlands
2017 Symposium Infectious diseases, Amsterdam, The Netherlands
2018 Conference on Retroviruses and Opportunistic Infections (CROI), Boston, United States of America
2018 Boerhaave course on Infectious Diseases, Noordwijkerhout, The Netherlands

Courses

2010 HIV Masterclass, Virology Education, Utrecht, The Netherlands
2011 Hepatitis Masterclass, Virology Education, Utrecht, The Netherlands
2012 Vaccination Masterclass, Virology Education, Utrecht, The Netherlands
2013 Basic clinical teaching, Maastricht UMC+, Maastricht, The Netherlands
2016 Basis course on Regulations and Organisation for clinical investigators (BROK), Dutch federation of University Medical Centers (NFU)
2017 Research Integrity, Erasmus MC, Rotterdam, The Netherlands
2018 Teach the teacher III, Rotterdam, The Netherlands

Teaching

2010-ongoing Supervising doctorate and medical students
2014-ongoing Education of residents Internal medicine and Medical Microbiology and Infectious Diseases
2014-ongoing Yearly elective course 2nd year medical students: Infections of the big city

Research grants

2015 ZonMW, A metabolomics approach to improve personalized medicine in patients chronically infected with HIV. Co-applicant.
2017 NVHB, Tenofovir toxicity: association with inosine triphosphate pyrophosphohydrolase activity and *ITPA* genotype

CURRICULUM VITAE

Nicole Chantal Peltenburg was born on July 2nd 1980 in The Hague, The Netherlands. After she graduated in 1998 from the Fioretticollege in Lisse, she studied Pedagogy and Educational Sciences at the University of Amsterdam, The Netherlands, for one year, which she finished cum laude. In 1999 she started medical school at the University of Leiden, the Netherlands. After receiving her medical degree in April 2006, she worked for one year as a senior house officer in Internal Medicine in the Máxima Medical Center, in Eindhoven, The Netherlands, under supervision of Prof. dr. H.R. Haak. In 2007, she started as a resident in the Maastricht University Medical Center in Maastricht, The Netherlands (Prof. dr. C.D.A. Stehouwer and Prof. dr. R.P. Koopmans) and specialized in Infectious Diseases (Dr. S.H. Lowe). She started her PhD in 2010 at the Erasmus Medical Center, Rotterdam, The Netherlands, under supervision of Prof. dr. A. Verbon in collaboration with Dr. J. Bierau and Dr. J.A. Bakker, of which the results are presented in this thesis. After her training as internist-infectious disease specialist in 2014, she started working at the Erasmus Medical Center, Rotterdam, The Netherlands. Chantal is married to Aswin de Jong, and together they have three daughters: Celine (2011), Aimée (2013) and Anique (2017).

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