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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