Resistance mechanisms to Asparaginase in ALL pediatric patients, investigation of genetic data

Report

Plain language summary. Asparaginase is a key medicine for the treatment of acute lymphoblastic leukemia, a common type of blood cancer in pediatric patients. Given its enzymatic activity, its effect is caused by the depletion of an amino acid, asparagine, that is essential for the growth of cancer cells.

The drug is characterized by a large variability in the outcome. Almost half of patients experience some toxic effects, from mild to severe, while others respond poorly to the treatment. Side effects, namely allergic reactions, acute pancreatic inflammation, blood cloths and liver toxicity, can affect the treatment with asparaginase and be life threatening for the patients that experience them. For this reason, variables that influence the toxic events occurrence and the efficacy with the drug were investigated in the literature. Longer intervals between asparaginase doses and genetic alterations that increase the binding between the drug and its antibodies can increase the risk of an allergic reactions.

Furthermore, some studies show that when the immune system of the patient is activated (e.g. during an infection), the drug permanence in the serum is decreased. Some adaptations of the cell metabolism can also impact the ability of the drug to kill the cancer cells. In addition, some patients have genetic alterations that predispose them to be affected by acute pancreatic inflammation, given the ability of the drug to cause direct pancreatic damage. Hepatic toxicity can also be favored by the presence of some genetic alterations in enzymes important for the lipid storage. Its is also important to consider that concomitant treatment with other drugs that have an hepatic toxicity, can increase the risk for hepatic damage.

It emerged that many different genetic alterations can be predictive and causative of some side effects, thus making them important to use in clinical practice. The future goal would be to implement genetic testing to identify such variants and use their presence to adjust the dosage of the patients accordingly. In order to do that, genetic variants known to affect the toxic events occurrence need to be find in the population of patients treated at Prinses Maxima Center and using a statistical analysis, understand the causal relations with the clinical outcomes (toxic events or efficacy). This project focused first on collecting the state-of-the-art knowledge on the variables that influence the outcomes from asparaginase treatments, including genetic variants, with the aim of writing a review article. Later, the project focused on a method to read the patient's DNA alterations that are known from literature to be associated with the asparaginase outcome, uncovering limitations and problems. To extract this information, a software called BCFtools was used. Such software allows to read files containing the DNA alterations of the patient and extract the DNA sequence in specific positions. Together with this, a search on the available clinical data normally collected at the hospital was performed.

Abstract. Asparaginase, a drug used for the treatment of pediatric acute lymphoblastic leukemia, shows a broad rage of outcomes, with almost 50% of patients experiencing toxic effects. Using a systematic method, a literature search was performed to unveil the state-of-the-art knowledge on the variables that can influence asparaginase outcome. In many studies, several genetic variants were found associated with treatment outcome. Genetic and clinical data of acute lymphoblastic leukemia patients (treated with asparaginase according to the protocol), were already available in the hospital database.

The aim of the project was understanding the information needed and eventual limitations to process genetic data, mainly wide exome sequencing data, to extract genetic variants. Furthermore, in view of a clinical implementation of genetic investigation to tailor the dosage of asparaginase, research on the necessary and available clinical data was performed. BCFtools, a software used to manipulate and extract information from genetic sequencing files, was used to extract patients' genotypes for several positions already associated with asparaginase outcome in literature. Considering the information retrieved from the literature search, a list of clinically relevant phenotypes was created, and the hospital database was searched for the available ones.

Association between the genotype and phenotype of the patient can be made, although a larger study that includes more patients would be needed, since many of the genetic variants associated with the drug's outcomes are rare. Some clinical outcomes are not available, thus requiring prospective studies to collect such laboratory data.

Characterization of outcomes determinants.

Literature review methods: A systematic search was conducted on two databases, PubMed and Embase. The aim of the search was to extract published papers that investigated determinants of asparaginase outcomes. Later, papers where first selected by title, then full text. 98 articles were selected.

Asparaginase is an enzyme used in the treatment of pediatric lymphoblastic leukemia (ALL). It catalyzes the splitting of asparagine into aspartic acid and ammonia. The decreased concentration of asparagine, an essential amino acid that the leukemia cells need in order to grow and are unable to synthetize, leads to apoptosis (see fig. 1) In order to achieve its therapeutic role, a minimal serum enzymatic activity of 100 IU/mL is needed. An activity below this threshold would not significantly deplete asparagine, thus leading to lower efficacy and positive minimal residual disease (MRD).

Asparaginase treatment shows a broad range of outcomes, with a high interpatient variability. Almost 50% of patients develop side effects to asparaginase. [1] Asparaginase hypersensitivity is a common side effect

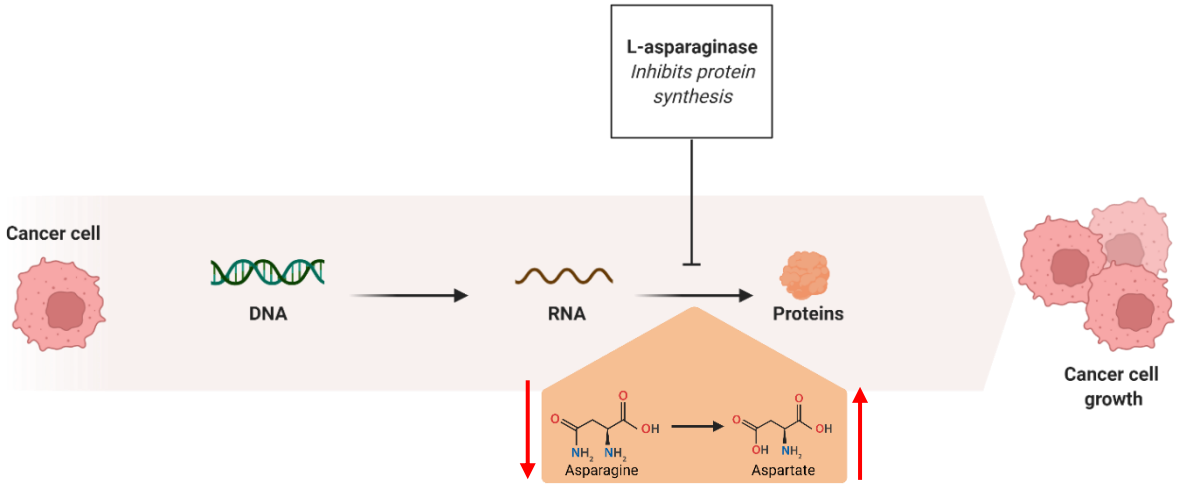

Fig. 1 Mechanism of action of asparaginase.

of the drug: different formulations of asparaginase also show different abilities to activate the immune system. In this context, therapeutic drug monitoring can discriminate between reactions mediated by antibodies, characterized by asparaginase inactivation, and allergic like reactions.

Furthermore, patients that experience hypersensitivity are switched from PEG Asparaginase (formulation commonly used) to Erwinia Asparaginase (Erwinase): Still 6% of patients rechallenged experience a second event, and of those exposed to Erwinaase 12-25% are fully discontinued from Asparaginase treatment [2], [3]. Several determinants can influence this side effect:

- Asparaginase-free intervals, intervals between two doses are a chance to produce antibodies against the enzyme. The longer the intervals, the more likely is the patient to experience hypersensitivity. [2]
- Polyethylenglycol (PEG) moiety antibodies prior the treatment, levels of IgM influence the clearance of asparaginase and can be predictive of a first dose reaction, that normally occurs in 1% of patients. [4]
- Corticosteroids can mask hypersensitivity reactions and or symptoms given the anti-inflammatory activity.

Furthermore, asparaginase-associated pancreatitis and thrombosis, although less frequent that hypersensitivity, can pose serious threats to the patient's health. In particular, asparaginase associated pancreatitis can cause patients to be admitted to intensive care units, with some patients developing chronic consequences (21% require insulin administration) or 46% develop a second even after re-exposure. [5] Several determinants can influence this side effect:

- Age, in particular older age is a risk factor for more serious complications.

- Peak dose intensity, (Native ASNase > 45000 IU/m^2) describe the dose in relation to the interval of administration and thus gives a clearer idea of the exposure. [6]
- Insulin resistance, defined as HOMA-IR (Homeostasis model assessment insulin resistance), is associated with more serious complications. Insulin resistance is a chronic inflammation of the pancreatic tissue and thus could predispose the patient to pancreatic inflammation. [7]

Thrombotic events can be deep venous thrombotic events or cerebral events. During such events the treatment with asparaginase has to be discontinued until full resolution and further continued coupled with anticoagulants. This side effect is thought to be caused by the therapeutic depletion of asparagine, which can result in decreased levels of anticoagulant proteins, in particular proteins C and S. [8] Several determinants were associated to this side effect:

- Older age, incidence 10-17.9 years group 15% vs 1-9.9 years group 4%. Some authors explain this association as older patients to be slower in recovering from anticoagulant proteins depletion.
- Non-O blood groups, where the AB0 antigen can mask the cleavage site of a pro coagulation factor (von Willebrand factor). [9]
- Corticosteroids, that are a known risk factor for thrombotic events, given their ability to increase procoagulant factors and decrease anticoagulants. [10]

Hepatotoxicity can affect up to 27% of patients treated with asparaginase. [11] This toxicity can impact the chemotherapy delivery, given the presence of several hepatotoxic medications. In case of hepatotoxicity, 38% of patients had a dose delay or modification of other medicaments. [11] Needless to say, other hepatotoxic compounds can increase the chance for this event, while higher exposure is associated with higher levels of transaminases. [12]

Not only toxic effects are recorded from asparaginase: patients can also experience lowered exposure to the drug or resistance to the treatment. The clearance of asparaginase influences the drug exposure, and several factors can impact on it. It is decreased in case of an immunity activation, thus during infections or during the initial phase of the treatment, given the high tumor load present. [13] On contrary, a stronger link between PEG and the enzyme can increase the half-life. [14] The association between toxic events the asparaginase enzymatic activity is driven mainly by the number of doses received. [15]

Resistance mechanisms, both intrinsic to the cancer cells and extrinsic, can hamper the efficacy of the treatment, given asparaginase importance as backbone of the induction treatment in the ALLTogether protocol. Given the frequency and the necessity of treatment truncation during such outcomes, more insights on the treatment are needed.

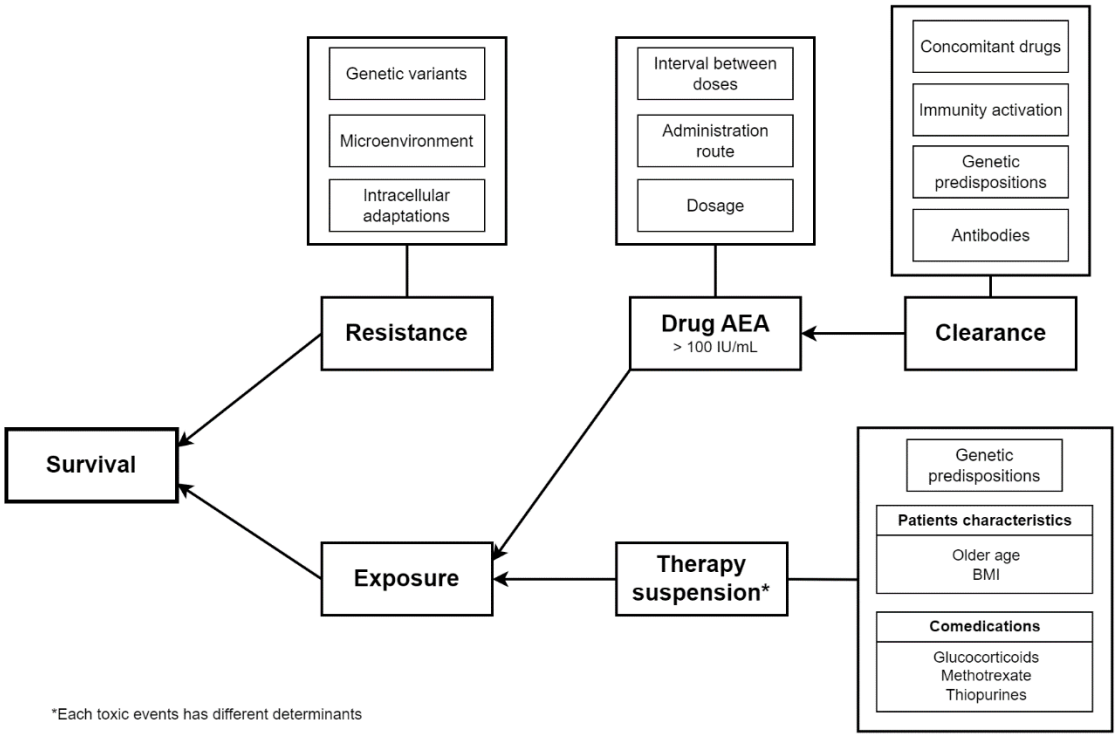

Figure 2 Summary of determinants of asparaginase outcomes. AEA: Asparaginase enzymatic activity. BMI: Body mass index

In Genome Wide Association Studies (GWAS) in large cohorts of pediatric ALL patients, several single nucleotide polymorphisms (SNPs) were associated to the phenotype relative to asparaginase treatment (Table 3).

Aim: To validate previously associated genetic variants and discover possible new genetic variants that could explain asparaginase outcomes by using Whole Exome Sequencing (WES) data.

Population:

Pediatric cancer patients (aged 1-18 years old) at Princes Maxima Center, with a diagnosis of lymphoma/leukemia that includes the usage of Asparaginase according to the protocol. Such patients have wide exome sequencing data available.

Inclusion criteria:

- Patients diagnosed with acute lymphoblastic leukemia (ALL)
- Treated with Asparaginase (any formulation)
- WES germline data available
- Informed consent signed

Methods

Study design: retrospective study design

Information required:

- WES data
- Hematology laboratory data and reporting of symptoms describing the outcomes (Asparaginase toxicity or efficacy) according to the definitions given by Ponte di Legno study group and/or CTCAE grading version IV.
- Collection time, since the opening of the hospital-up till now = 2018 - 2022
- Patients' demographics

Primary parameters/endpoints

- Phenotypes
- Genotypes

Phenotypes

Definitions: Ponte di legno criteria for toxic outcomes definition (*Schmiegelow 2016*) and grading according to CTCAE version IV.

- *Hypersensitivity*, flushing, rash, urticaria, drug fever, dyspnea, symptomatic bronchospasm, oedema/angioedema, hypotension and/or anaphylaxis. If this is associated with Enzymatic activity levels below lower limit of quantification is a proper allergic reaction. Otherwise, symptoms only indicate an allergic like reaction. If clinical symptoms are not manifesting but the enzymatic activity levels are below lower limit of quantification, then silent inactivation is present.
- *Acute asparaginase-pancreatitis (AAP)*, at least two of the three following symptoms must be present. Serum amylase and/or lipase ≥3x Upper normal limit (UNL), abdominal pain, imagine finding of acute pancreatitis.
- *Thromboembolism (TE)*, venous or arterial thromboembolic event. Requires imaging confirmation for grading equal or superior than 2.

- *Hepatotoxicity*, High hepatic transaminases (AST or ALT) or hyperbilirubinemia. Severe events are defined as likely to impact chemotherapy delivery. Grade ≥4 transaminitis (AST or ALT>20× ULN), Grade ≥3 hyperbilirubinemia (>3× UNL).
- *Hypertriglyceridemia (HTG)*, levels > 150 mg/dL, higher than the UNL.

Phenotypes – Lab data	Type of phenotype	Indicating	Additional information	Present
Hypertriglyceridemia	Final/Intermediate	HTG	Serum triglycerides/cholesterol	no
ASNase sensitivity	Final	Resistance	On patients' blasts	no
MRD (day 29 and 71)	Intermediate	Efficacy		Yes
Therapeutic drug monitoring	Intermediate	Hypersensitivity		no*
ASNase antibodies	Intermediate	Hypersensitivity		no
Pancreatic enzymes	Intermediate	AAP	Lipase/amylase serum	Yes
Abdominal Imaging	Intermediate	AAP		yes
Reported pain/fever	Intermediate	AAP		Yes
Coagulation factors	Intermediate	TE	Partial thromboplastin time, fibrinogen	Yes
Hepatic enzymes	Intermediate	Hepatotoxicity	Transaminase (AST, ALT)/Bilirubinemia	yes
Serum albumin	ND	NA		yes
Infection	Final	NA	Available antibiotics, or C-reactive protein	yes
Renal function	Final	NA	Estimated glomerular filtration rate (eGFR), Creatinine	yes

*Table 1 Intermediate phenotype characterize other outcomes; Final phenotype is the outcome of interest. *not all patients, it was implemented later. NA, not applicable.*

Additional patient data: Gender, age at diagnosis or age at start treatment, BMI, start date of asparaginase treatment, tumor type, ASNase dosage received and possible dose reductions, formulation used and administration route.

On a later stage information on mRNA data and DNA methylation can also be used. Particularly, DNA methylation can be important to assess silencing of some genes important for ANSase resistance mechanisms (eg: GCN2 promoter).

Notes:

- ALL patient sequencing is not performed any longer. In the central subject registry (CSR) of the Princess Maxima Center there are 55 patients with such diagnosis. It is expected that all these patients have been exposed to ASNase therapy. According to the raw data obtained from the CSR database, 51 out of 58 samples have Wide exome sequencing (WXS) germline SNV detection, while all 58 patients have the WGS Germline SNV Detection,

Gene	SNP ID	Phenotype	Location	P-value and effect size	Ref.
SOD2*	rs4880	Hepatotoxicity	chr6:159692840	CC genotype P=0.018, OR 2.6	Alachkar et al. 2017
PNPLA3	rs738409 (C > G)	Hepatotoxicity	chr22:43928847	2.52E-8, 1.29	Liu et al 2017
GRIA1	rs4958351	Hypersensitivity	chr5:153790814	1.81E-5, OR 1.93	Chen et al 2010
NFATC2	rs6021191 (A > T)	Hypersensitivity	chr20:51419700	4.1E-8 , OR 3.11	Fernandez 2015
HLA-DRB1*07:01-DQA1*02:01-DQB1*02:02	rs28383172 and rs7775228	Hypersensitivity	chr6:32598202 and chr6:32690302		Kutszegi et al 2022
SLC7A13	rs9656982 (A > G)	Hypersensitivity	chr8:86214471	GG genotype P=0.03 OR 8.8	Abaji et al 2017
YTHDC2	rs75714066 (G > C)	Hypersensitivity	chr5:113553673	GC genotype P=0.005 OR 3.3	Abaji et al 2017
CNOT3	rs73062673 (T > C)	Hypersensitivity	chr19:54151243	4.68E-8, OR 3.7	Højfeldt et al 2019
NFATC2	rs62228256 (C > T)	AAP	chr20:51837908	5.18E-8, OR 3.75, not replicated	Wolthers et al., 2019
ADAMTS17	rs72755233 (G > A)	AAP	chr15:100152748	GA genotype P=0.002 OR 5.9	Abaji et al 2017
ULK2	rs281366	AAP	chr17:19897081	5.8E-7, OR 6.7	Wolthers et al., 2017
RGS6	rs17179470	AAP	chr14:72086035	1.27E-6, OR 4.39	Wolthers et al., 2017
CPA2	rs199695765	AAP	chr7:130269008		Liu et al., 2016

PRSS1	rs10273639 (C > T)	AAP	chr7:142749077	Replicated p=0.04 HR 0.69	Wolthers et al., 2019
PRSS2	rs13228878 (A > G)	AAP	chr7:142765617	Replicated p=0.03 HR 0.68	Wolthers et al., 2019
IL16	rs11556218 (T > G)	AAP Thrombosis	chr15:81305928	Genotype TG P=0.009 OR 7.9	Abaji et al 2017
MYBBP1A	rs3809849 (G > C)	AAP Thrombosis Hypersensitivity	chr17:4555303	Genotype GC P=0.0005 OR 7.9	Abaji et al 2017
ALOX15B	rs1804772	Thrombosis	chr17:8048687	3.95E-7, OR 8.1	Mateos et al 2020
KALRN	rs570684	Thrombosis	chr3:124403999		Mateos et al 2020
SPEF2	rs34708521 (G > A)	Thrombosis	chr5:35670201	Genotype GA P=0.03 OR 6.3	Abaji et al 2017
RIN3	rs3742717 (C>T)	Thrombosis	chr14:92652323	Genotype TT P=0.02 OR 14.6	Abaji et al 2017
ABCC4	rs34839857 (insertion)	AAP	chr13:95028069-95028070	0.01, OR 3.33	Bartram et al 2021
ABCC4	rs4148513	AAP	chr13:95138099	7.31E-9, OR 47.20	Bartram et al 2021

*Table 2 SNPs to test for association with asparaginase toxicity or efficacy outcomes *Identified in adult population associated to asparaginase hepatotoxicity.* [16]

Additional genetic information: allele frequency in general population and in cohort (if applicable).

Genetic data and bioinformatics tools

The analysis was conducted on 58 Biomaterial IDs files. Each file equals to one patient, except from 4 patients that had a double sample, a thus 2 different Biomaterial IDs.

There are two types of files available from wide exome sequencing.

- .cram files contain all reads are used with the reference genome Hg38. They are useful to check when doubts about the power of the sequencing occur. As mutation confirmation.
- .vcf files, variant calling files, contain all variants from the reference genome. They could contain false positives, since they are not filtered, meaning that even if only one read reports a mutation out of 100, it will still be reported as mutation. Depth, indicates how many reads for the sequence, while Allele frequency, indicates the ratio allele read/total reads.

The corresponding genotype in the positions that hold such polymorphisms (table 2) were investigated in the cohort. This was done with the help of the BcfTools software. Data were manipulated to extract the genotype at each position in table 2.

In detail, BcfTools is a software that allows processing and analysis of variant files (VCF or BCF). The steps that were taken to extract the data.

1) From the large cohort of the sequencing data from all tumor types, filenames that were associated with a diagnosis of ALL were isolated through the rsync command that allows a list of file names as input;
2) The files then obtained were first indexed with BCFtools, using the command *BCFtools index *vcf.gz*, that allows to index all VCF files contained in the directory of interest. This step is necessary given that the index files are necessary to further manipulate the data and read the single files to check the further steps.
3) All files were merged into one VCF file. This was done through the bcftools merge command, that allows a .tsv file (that can be created from google sheet) as an input. This file can contain information about regions/positions that are of particular interest;
4) Once obtained the VCF file containing the information for all the cohort and all positions of interest, the information there contained can be manipulated.
5) The VCF file was manipulated using the query function of BCFtools that allows to format the output information as needed. The format used is indicated in the following command:
   bcftools query -f '%CHROM:%POS\n[\n%SAMPLE\t[GT=%GT]]\n' Merged.vcf.gz > output1.tsv
   This allows to extract the information of chromosome, position for each sample (patient) and the genotype for that position for that sample.
6) The data obtained was then manipulated and cleaned on Excel.

Notes

- Submitting a .tsv file to filter out the variants. If a variant is not called in any of the samples of the cohort, then nothing gets reported on the output. For this reason, the variants from the list that were investigated that are not present in the genotypes file are not present (the patients are homozygous for the reference allele)
- *How do I make sure to be using the germline data available*? “02. Disease status” the entry *normal* indicates that the biosource sample was not a tumor. Result files from the GSNV workflow (these will have only one Biomaterial Id in their filename), are definitely germline variants.
- *There are 4 cases of patients with the sequencing done two times*. 3 of them have a different biosource ID, that can indicate that two different tissues, but one has the same biosource ID. I would expect anyway that the two sequencing files have the same SNP individuated, but that was not the case. They

should mostly overlap. On the other hand I know that VCF files can contain one SNV even if only one alternative read was detected, for that reason it is important to check the depth of the reads. Trying with the isec command, many different variants are called only in one of the two files.

- Interestingly, each file (each biosource ID correspondent file) contains two samples, .variant and .variant2. If a call is made for a position, it is always present in the first one. But interestingly, at a position a deletion occurred. For that reason it has been reported in the VCF file, but at the same position another SNP is present, within the same file. *How can a file contain both a deletion and a SNP, does that mean that one allele is the mutation and the other is deletion?*

Results and example of patient

Gene, SNP ID, phenotype associated	REF	ALT	Genotypes extracted
SLC7A13, rs9656982, Hypersensitivity	A	G	12/58 Heterozygous
YTHDC2, rs75714066, hypersensitivity	G	C	4/58 Heterozygous
PRSS1, rs10273639, AAP	T	C	22/58 Heterozygous 19/58 Homozygous alt allele
PRSS2, rs13228878, AAP	G	A	2/58 Homozygous alt allele
ADAMTS17, rs72755233, AAP	G	A	11/58 Heterozygous 1/58 Homozygous alt allele
ALOX15B, rs1804772, thrombosis	C	A	6/58 Heterozygous 3/58 Homozygous for the alt allele
SPEF2, rs34708521, Thrombosis	G	A	9/58 Heterozygous (1/4 patient with double sample are discordant results between samples)
IL16, rs11556218, AAP thrombosis	T	G	11/58 Heterozygous (2/4 patient with double sample are discordant)
KALRN, rs570684, Thrombosis	T	C	1/58 Homozygous for the alt allele
SOD2, rs4880, Hepatotoxicity	A	G	29/58 Heterozygous (1/4 patients with double samples are concordant) 16/58 Homozygous for the alt allele (2/4 of patients with double samples are concordant)
PNPLA3, rs738409, hepatotoxicity	C	G	18/58 Heterozygous 7/58 Homozygous for the alt allele (1/4 of patients with double samples are concordant and 1/4 of patients with double samples are discordant)

Table 3 Genotypes within the cohort

Table 2 reports the genotypes found within the cohort using the methods above described. Interestingly, when analyzing the genotype in the pool of SNPs found in literature, patients that have a double Biomaterial ID associated (meaning that have two samples taken) are not always concordant. The SNPs reported in table 1 and not found here have been resulted as heterozygous for the reference allele.

Example of patient: Clinical data was extracted for a patient in the cohort. According to the medical report, the patient experienced AAP. In that case the genotype is as following:

Gene, phenotype associated	SLC7A13, Hypersensitivity	YTHDC2, Hypersensitivity	PRSS1, AAP	PRSS2, AAP	ADAMTS1, AAP
Patient genotype	Homozygous for the reference allele	Homozygous for the reference allele	Heterozygous	Homozygous for the reference allele	Homozygous for the reference allele

SPEF2, Thrombosis	IL16, AAP Thrombosis	KALRN, Thrombosis	SOD2, Hepatotoxicity	PNPLA3, Hepatotoxicity
Homozygous for the reference allele	Homozygous for the reference allele	Homozygous for the reference allele	Homozygous for the alternative allele	Homozygous for the reference allele

For this patient, data to collect:

- Serum amylase or lipase
- Body temperature
- Imaging confirmation

Other covariates:

- Age
- Dosage of ASNase
- Clearance
- Body weight
- Infections

Discussion and future perspectives

It is necessary to confirm that the steps taken do not lead to missed information. In particular, the accuracy of the genotype extracted should be evaluated. Furthermore, understanding how to deal with samples referring to the same patients with different genotypes from the extraction is necessary.

What type of association analyses can be performed? To answer this the **statistical power** of the cohort is needed (probability to find a variant if actually present). It needs to be at least 0.8, otherwise the study doesn't make sense. To calculate it, the allele frequency of the alleles of interest, within the general population or the selected cohort, and genotype relative risk are needed.

Calculating the statistical power has to be done for each interesting variant to look into. The suggested software for such analysis is PLINK 1.9.

Some information relative to the clinical outcome is missing, in particular ASNase antibody concentrations, serum triglycerides, sensitivity to ASNase and levels of ASNase activity. Prospective data collection is necessary to collect this information.

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