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**Clinical, biochemical and molecular phenotype of congenital disorders of glycosylation:
long-term follow-up**

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Abstract:

Background: Congenital disorders of glycosylation (CDG) result from defects in the synthesis of glycans and the attachment of glycans to proteins and lipids. Our study aimed to describe the clinical, biochemical and molecular findings of CDG patients, and to present the long-term follow-up.

Material and methods: A single-centre study (1995-2019 years) of patients with congenital disorders of N-glycosylation and combined N- and O-hypoglycosylation, diagnosed based on the serum transferrin (Tf) and apolipoprotein C-III (apoC-III) isoforms analysis, and confirmed molecularly, was performed.

Results: Among 32 patients included into the study, 24 had type I Tf isoform profile, in 12 of them deficient PMM2 activity was detected. Three patients were diagnosed with ALG13-CDG; serum Tf isoform profile was normal in one of them, in one other was indicative for type I. Four patients had type II Tf isoform profile. The phenotypic and genotypic spectrum of 32 patients with CDG during long-term (in some cases over 20 years) observation was characterised and several measurements of serum Tf isoforms taken. Statistical analysis revealed strong negative correlation between Asialo-Tf and Tetrasialo-Tf, as well as between Disialo-Tf and Tetrasialo-Tf. Positive correlation was shown between Tetrasialo-Tf and Pentasialo-Tf. Within type I CDG, no difference in % Tf isoforms was revealed between PMM2-CDG and non-PMM2-CDG patients. However, these two groups differed significantly in the such diagnostic features as: cerebellar ataxia, failure to thrive, hypothyroidism, pericardial effusion, cardiomyopathy, inverted nipples, prolonged INR.

The effect of treatment with mannose in 2 patients with MPI-CDG were assessed and we found that % of Asialo-Tf, Monosialo-Tf, and Disialo-Tf was significantly lowered, whereas Tetrasialo-Tf and Pentasialo-Tf rose, coming closer or falling into the reference range.

Conclusions: The novel finding was an abnormal Tf IEF pattern in two ALG13-CDG patients and normal in one ALG1-CDG patient. Clinical manifestation of presented CDG patients was similar to that reported in the literature. Mannose supplementation in MPI-CDG patients, as well as galactose supplementation in PGM-CDG patient improved patients' clinical picture and Tf isoform profiles.

Key words: glycosylation, congenital disorders of glycosylation, serum transferrin isoforms, follow-up

List of abbreviations:

ALT – alanine aminotransferase,
ApoC-III – apolipoprotein C-III,
AST – aspartate aminotransferase,
CDG – congenital disorders of glycosylation
IEF – isoelectrofocusing,
LLO – lipid-linked oligosaccharide,
INR – international normalized ratio,
MPI – mannosephosphate isomerase,
NGS – next-generation sequencing,
NIHF – non-immune hydrops foetalis,
PGM1 – phosphoglucomutase 1,
PMM2 – phosphomannomutase 2,
Tf – serum transferrin,
WES – whole-exome sequencing.

1. Background

Congenital disorders of glycosylation (CDG), first reported in 1980, result from defects in the synthesis of glycans and the attachment of glycans to proteins and lipids [1]. According to the current nomenclature, CDG can be classified into defects in protein N-glycosylation, O-glycosylation, glycosphingolipid and glycosylphosphatidylinositol anchor glycosylation defects, and multiple glycosylation pathways defects. Since the description of phosphomannomutase two deficiency (PMM2-CDG), more than 130 other CDG subtypes, have been reported [2-3].

So far, single case reports and case series regarding various CDG have been published, but the data regarding follow-up are sparse in the literature. Our study aimed to describe the clinical, biochemical, including serum Tf isoform analysis, as well as molecular features of patients diagnosed with CDG in one referral centre, and to present the long-term follow-up.

2. Material and methods

During 1995-2019 years, a total number of 22063 serum Tf isoform analysis, have been done in our Institute. Among this group, 32 patients with molecularly confirmed congenital disorders of N-glycosylation and combined N- and O-hypoglycosylation, diagnosed and followed-up have been recruited to the study. The chart review of patients' medical records concerning the demographics, first presented signs and symptoms, age at diagnosis, diagnostics methods (described below), clinical outcome as well as biochemical (serum Tf isoforms, aspartate (AST) and alanine (ALT) aminotransferases, international normalized ratio (INR), and molecular data were collected. Ethical approval was obtained from the Children's Memorial Health Institute Bioethical Committee, Nr 23/KBE/2020, Warsaw, Poland.

Serum Tf isoforms were analyzed by isoelectrofocusing (IEF) agarose gel electrophoresis according to our modification [4-5] of the method described by Van Eijk et al. [6]. Serum Tf consists of the mixture of isoforms and has two iron binding sites, which should be saturated with iron (20 μ l serum with 80 μ l 0,9% NaCl, 2 μ l 10 mM Fe(III) citrate and 2 μ l 0.1 M NaHCO₃). Transferrin migrates through and 1% agarose gel (45 mg of agarose were added to 4.5 ml of distilled water) with 5% ampholines in a pH range of 5.0-7.0 on a Multiphore 2117 apparatus (LKB) with a modified electrode lid. Tf isoforms were visualized by immunofixation (200 μ l polyclonal rabbit anti-human transferrin serum) and 0.5% Coomassie Brilliant Blue solution staining, as presented in **Figure 1**. The percentage of Tf fractions was

assessed densitometrically and carbohydrate deficient transferrin value (% CDT) was measured as a sum of asialo-, monosialo-, and disialoTf isoforms.

Serum O-glycoprotein apolipoprotein C-III (ApoC-III) isoforms were analyzed by isoelectrofocusing polyacrylamide gel electrophoresis according to the method described by Wopereis et al. [7]. The activity of phosphomannomutase (PMM2; EC 5.4.2.8) and mannosephosphate isomerase (MPI; EC 5.3.1.8) in fibroblasts were determined according to the method of Van Schaftingen and Jaeken [8]. Phosphoglucomutase 1 (PGM1) enzyme activity was assayed spectrophotometrically on cell extracts [9-10]. Lipid-linked oligosaccharide (LLO) profiles in fibroblasts were analyzed by high-performance liquid chromatography (HPLC) [11-12]. Serum dolichols were analyzed after lipid extraction, and dolichol and isoprenol species (18-21 isoprenol P-P units) were separated by high-performance liquid chromatography in a reverse phase column and detected spectrophotometrically [13].

Molecular analysis, using direct sequencing of single genes, panel-based next-generation sequencing (NGS), or whole-exome sequencing (WES) was applied. The nomenclature of identified variants and patients' genotype follows the Human Genome Variation Society guidelines (HGVS v 2.0, www.hgvs.org/mutnomen) and referral according to cDNA and protein sequences of various CDG genes followed the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk).

An extended statistical analysis was performed. First, in order to find correlations between % of Tf isoforms, we calculated Pearson's linear correlation coefficient r between all Tf isoforms. Next, we compared the subcohort of PMM2-CDG-I and non-PMM2-CDG-I patients in terms of the mean of % Tf isoforms. We also performed chi-squared test for independence to establish if diagnostic variables, such as muscle hypotonia, hypothyroidism, nystagmus, etc., are independent of the type of CDG-I disease. Finally, we investigated the effect of treatment administered in three patients on the % Tf isoforms. We compared means (or single values) measured before treatment with the means of measurement taken after the first administration of mannose administration (in one patient with CDG-I/CDG-II) or galactose administration (two patients with non-PMM2-CDG-I).

The alpha level of significance was chosen 0.05. For computations we used statistical software "R".

3. Results

3.1. Overall characteristics

Twenty four patients had type I Tf isoform profile (CDG- I), four patients had type II Tf isoform profile (CDG-II), and one patient had a mixed type (CDG-I/CDG-II). Three patients (from one family) with type II showed an alteration in the apoC-III isoform profile (increased apoCIII-1, decreased apoCIII-2), indicative of a combined N- and O-glycosylation defect. Three patients with ALG13-CDG were diagnosed by whole-exome sequencing; serum Tf isoform profile was normal in Patient 14 in the other (Patient 13) was indicative for CDG-I, in the third (Patient 15) slightly elevated disialo-Tf. One patient with ALG1-CDG (Patient 18) was diagnosed based on array comparative genomic hybridization in which chromosome 16p13.3 deletion involving *ALG1* gene was found.

A deficient PMM2 activity was detected in 12 patients with CDG-I. In three other patients, MPI activity was deficient. Serum dolichol profiles were studied in three patients with CDG-I, revealing a slight increase in the levels of isoprenols (chain length 18-20) from all the patients affected with SRD5A3-CDG.

Finally, 12 patients with PMM2-CDG, three patients with ALG13-CDG, three patients with ALG1-CDG, one patient with ALG3-CDG, three patients with MPI-CDG, one patient with PGM1-CDG, four patients with SRD5A3-CDG, one patient with DPAGT1-CDG, three patients with ATP6AP1-CDG, and one patient with ATP6V0A2-CDG, were recruited to the study. Some of them have been previously reported as single case reports or case studies [10, 14-15]. Detailed characteristics is presented in *Supplementary Table S1*.

3.2. PMM2-CDG patients

There were 12 patients with PMM2-CDG, nine males and three females. All patients presented with an early-onset (within 2 years of age) of the disease. Non-immune hydrops foetalis (NIHF) was present in two patients.

3.2.1. Presentation at diagnosis

The frequency of observed symptoms and features in the cohort of PMM2-CDG patients is given in **Table 1**. It is compared to the frequency of symptoms in the cohort of non-PMM2-CDG.

Among eleven of diagnosed patients, 13 various pathogenic variants in the *PMM2* gene (NM_000303.2) have been identified, including 10 missense mutations, 2 intronic mutations

and 1 frame-shift mutation. The most frequent mutations were p.V231M (22%) and p.R141H (22%).

3.2.2. Serum Tf IEF

Serum Tf IEF in all patients showed elevated asialo- and disialo-Tf isoforms. Eleven out of twelve PMM2-CDG patients had low tetrasialo-Tf. Levels of all seven Tf isoforms of patients with PMM2-CDG are presented in **Figure 2** (first twelve dots, blue).

3.2.3. Follow-up

Among 12 patients diagnosed, 11 are followed-up. The mean time of follow-up was approximately 8 years (range: 8 months – 18 years). At the last follow-up, 10 patients were alive while two others (Patient 6 and 7) had died at the age of 9 and 2 months, respectively. Patient 6 demonstrated prenatally NIHF; the exact cause of death is not known. Patient 7 had a neurovisceral form of the disease with liver and cardiac involvement, he was on mechanical ventilation since the second week of life. *Post mortem* examination revealed liver fibrosis. Improvement of motor skills was observed in the majority of patients except for three alive patients with cardiac features. Due to massive pericardial effusion, two of them required pericardiocentesis, and pleural-pericardial window formation, finally. In two, feeding by a nasogastric tube was needed.

3.3. Non-PMM2-CDG patients

There were 20 patients, including three ALG13-CDG, three ALG1-CDG, one ALG3-CDG, three MPI-CDG, one PGM1-CDG, four SRD5A3-CDG, one DPAGT1-CDG, three ATP6AP1-CDG, and one ATP6V0A2-CDG.

All the patients presented with an early-onset (within 2 years of age) disease. One patient diagnosed with ATP6AP1-CDG deficiency had NIHF.

3.3.1. Presentation at diagnosis

Neurological involvement was noted in the majority of non-PMM2-CDG patients besides MPI-CDG, ATP6AP1-CDG and ATP6V0A2-CDG. Muscle hypotonia and psychomotor delay were the most common features. Seizures occurred in 5 patients, including three ALG13-CDG and two ALG1-CDG. All patients with ALG13-CDG presented with an early-

onset (between 2 and 4 months of age) drug-resistant epileptic encephalopathy. One ALG1-CDG patient had seizures in the first week of life with subsequent sudden cardiac arrest and diagnosis of brain oedema in computed tomography.

In brain MRI, cerebellar hypoplasia in four SRD5A3-CDG patients, brain atrophy in one ALG13-CDG patient, pachygyria and polymicrogyria in one ATP6V0A2-CDG patient, and hypomyelination of the brain in one DPAGT1-CDG patient were found.

Congenital microcephaly was seen in six patients, two ALG13-CDG, three ALG1-CDG, and one ATP6V0A2-CDG.

Six patients had hepatomegaly (one ALG1-CDG, one ATP6AP1-CDG, one PGM1-CDG, and three MPI-CDG) while thirteen presented elevated serum transaminases (one ALG1-CDG, three MPI-CDG, one PGM1-CDG, four SRD5A3-CDG, one DPAGT1-CDG, and three ATP6AP1-CDG). Synthetic liver dysfunction was noted only in 2 patients (one SRD5A3-CDG, one ALG1-CDG).

Coagulation factor abnormalities, such as low serum protein C, S and antithrombin levels, were found in 12 out of 15 patients (in five patients there were not analyzed).

Skin features were present in one patient diagnosed with ATP6V0A2-CDG in the form of cutis laxa.

Endocrine features were observed in one patient diagnosed with PGM1-CDG in the form of hypogonadotropic hypogonadism with delayed puberty.

Among 15 of patients, 12 various pathogenic variants in the CDG associated genes have been identified, including 11 missense mutations and 1 frame-shift mutation. All of them were previously reported.

The frequency of observed symptoms and features in the cohort of non-PMM2-CDG patients is given in **Table 1**.

3.3.2. Serum Tf IEF

Serum Tf IEF was abnormal in 18 out of 20 patients. One of the patients with ALG13-CDG and one with ALG1-CDG had normal Tf IEF while other patients with ALG1-CDG had significantly increased asialo- and disialo-Tf. Tf isoform showed slightly elevated asialo- and disialo-Tf in the first patient with ALG13-CDG and slightly elevated disialo-Tf in the third patient with ALG13-CDG. All patients with MPI-CDG presented a decrease in percentage of CDT on mannose supplementation. Four patients with SRD5A3-CDG showed similar Tf isoform values and %CDT within 40,1 – 47,1. Patient with DPAGT1-CDG had a typical type I Tf isoform profile with %CDT 33,2.

Four patients had type II Tf isoform profile: three with ATP6AP1-CDG, and one with ATP6V0A2-CDG. ATP6V0A2-CDG patient showed mildly elevated disialo-, elevated trisialo-Tf and decreased tetrasialo-Tf. Patients with ATP6AP1-CDG had elevated disialo- and trisialo-Tf. Tetrasialo-Tf was decreased in all four patients. Levels of all seven Tf isoforms of patients with non-PMM2-CDG are presented in **Figure 2**. Yellow dots represent non-PMM2-CDG-I patients and green dots represent non-PMM2-CDG-II patients.

PGM1-CDG patient showed a mixed type I/II Tf isoform profile with significantly increased asialo-, monosialo- and disialo-Tf and highly decreased tetrasialo-Tf. Tf isoforms of this patient are depicted with purple dots in **Figure 2**. The improvement of Tf isoform on galactose supplementation was noted. Measurements before the start of treatment and after the start of treatment with respective means are given in **Figure 4**.

3.3.3. Follow-up

Among 20 patients, 19 are followed-up. The mean time of follow-up was approximately 7 years (range: 1 month – 19 years). At the last follow-up, 15 patients were alive while 4 others had died (two ALG1-CDG, one PGM1-CDG, one DPAGT1-CDG).

Patients with ALG1-CDG presented a severe phenotype leading to death in the first months of life.

MPI-CDG patients demonstrated the improvement of clinical features on mannose supplementation.

Patient with PGM1-CDG manifested with progressive cardiac insufficiency and liver impairment (hepatomegaly, elevated serum transaminases) since 4 years of age. Diagnosis of PGM1-CDG was established at 10 years of age, the galactose supplementation was started just at the age of 16 years. The patient had died at 19 years due to cardiac failure.

Three out of four patients with SRD5A3-CDG showed an improvement of motor skills and speech development, the other one (Patient 26) demonstrated spastic tetraparesis and needed gastrostomy insertion.

The progressive disease course was observed in three males affected with ATP6AP1-CDG. Sensorineural hearing loss up to total deafness as well as progressive hair loss up to total alopecia was observed in all of them. Two of them (Patient 30 and 31) also developed glomerular proteinuria, during follow-up.

3.4. Statistical analysis

3.4.1. Correlations between isoforms

We calculated Pearson's linear correlation coefficients r between all Tf isoforms, finding that the most strong, negative correlations exists between asialo-Tf and tetrasialo-Tf ($r=-0.86$), disialo-Tf and tetrasialo-Tf ($r=-0.8$), disialo-Tf and pentasialo-Tf ($r=-0.77$). The only strong and positive correlation was revealed between tetrasialo-Tf and pentasialo-Tf ($r=0.78$). These results differ only slightly between the subcohort of PMM2-CDG and non-PMM2-CDG patients. Detailed results are given in *Supplementary Table S2*. Four strongest correlations between Tf isoforms are depicted in **Figure 3**.

3.4.2. Comparison of PMM2-CDG and non-PMM2-CDG

We performed t-test (or its variant - Welsh test, where appropriate) to establish the statistical significance of the differences in means of Tf isoforms between PMM2-CDG and non-PMM2-CDG. We found that none of the Tf isoform differ significantly between these two groups of patients. The means of % Tf isoforms and respective p-values are given in *Supplementary Table S3*.

PMM2-CDG and non-PMM2-CDG patients were also compared in terms of presented symptoms. Using chi-squared test for independence, we found that the variable "PMM2-CDG or non-PMM2-CDG" is dependent of the following diagnostic variables: cerebellar ataxia, failure to thrive, hypothyroidism, pericardial effusion, cardiomyopathy, inverted nipples, prolonged INR. The whole set of analysed symptoms with the p-values of the chi-squared tests are given in **Table 1**.

3.4.3. Assessment of treatment

There were three patients for whom data on % Tf isoforms were available before and during treatment. One MPI-CDG patients had one measurement taken before the start of treatment and eleven during the treatment. We compared the mean of the results obtained during treatment with the value measured before the start of treatment using one-sample t-test. All % Tf isoforms changed (except for trisialo-Tf), bringing the values closer to the reference range. Asialo-, monosialo- and disialo-Tf drop significantly (more than or approximately half), whereas tetrasialo- and pentasialo-Tf rise significantly and meet their respective reference ranges.

The second MPI-CDG patient had four measurements taken before the start of the treatment and twelve taken during treatment. Similarly, asialo-, monosialo-, disialo- and trisialo-Tf % drop, whereas tetrasialo- and pentasialo-Tf % rise.

The PGM1-CDG patient had seven measurements performed before the start of treatment, and eleven on treatment. Here we performed t-test to compare the means before and during treatment, finding similar results than in MPI-CDG patients: asialo-, monosialo-, and diasialo-Tf are lowered, whereas tetra- and pentasialo-Tf rise enough to fall into the reference range.

Results for all three patients, along with reference ranges for comparison, and p-values are presented in *Supplementary Table S4*.

We also depict the effect of galactose supplementation in PGM1-CDG patient in **Figure 4**. An analogous visualisation for patients with MPI-CDG who had four measurements of % Tf isoforms taken before the start of mannose supplementation is given in *Supplementary Figure S1*.

4. Discussion

The paper presents the phenotypic and genotypic spectrum of 32 patients with CDG during long-term (over 20 years) observation and single-centre experience.

PMM2-CDG was the most common (40%) CDG identified in our study, similarly to the literature [16-17]. Among the N-hypoglycosylation disorders, ALG6-CDG is reported as the second, while ALG1-CDG as the third most frequent type [18]. We did not identify any ALG6-CDG patients, whereas SRD5A3-CDG was the second most frequent type. ALG1-CDG, ALG13-CDG and MPI-CDG were comparably frequent.

Prenatal presentation (NIHF) was observed in three (9%) of our patients, including two PMM2-CDG (one died at two months of age). In a recent review by Makhamreh et al. (2020), the most common CDG associated with NIHF were PMM2-CDG, ALG9-CDG, and ALG8-CDG, which follows the line of our results [19]. Our paper is one of the first to report NIHF associated with ATP6AP1-CDG. Recently, thickened nuchal translucency and large fluid filled space with septations along the fetal spine have been described during first trimester ultrasound in ATP6AP1-CDG fetus [20].

CDG patients frequently present with neurological involvement [21]. The main neurological symptom in our patients remained psychomotor retardation and cerebellar ataxia with cerebellar hypoplasia on brain MRI scans, observed in 83% of PMM2-CDG patients and all SRD5A3-CDG patients. This finding confirms the thesis that the cerebellum is regularly involved in PMM2-CDG and SRD5A3-CDG [14, 25-27]. Epilepsy is also a common

symptom (almost one-third of patients with PMM2-CDG in the study by Monin et al. [22]), in our cohort it was observed in ALG13-CDG patients. There are others CDG in which epilepsy is severe and difficult to control, like DPM1-CDG, DPM2-CDG, MPDU1-CDG, ALG2-CDG, ALG12-CDG, ALG8-CDG, ALG9-CDG, ALG11-CDG and RTF1-CDG [23-24].

Liver involvement was present in about 22% of all CDG in the last review by Marques-da-Silva et al. (2018) [28]. In our cohort, the predominant liver phenotype was observed in MPI-CDG patients, while other CDG like PMM2-CDG, ATP6AP1-CDG were associated with liver disease. Up to now, 14 patients have been reported with the X-linked ATP6AP1 deficiency and the key features were immunodeficiency and liver involvement ranging from a mild elevation of serum transaminases to liver failure [15, 29-30].

Our results are in agreement with the thesis that selected CDG have unique characteristics, which may facilitate and shorten their recognition. These include: connective tissue involvement in ATP6AP1-CDG, midline malformations in PGM1-CDG, chronic diarrhoea in MPI-CDG, inverted nipples and abnormal fat distribution in PMM2-CDG, cataract/coloboma in SRD5A3-CDG, cerebellar hypoplasia in PMM2-CDG, SRD5A3-CDG [31]. Moreover, some of CDG are noted to manifest specific craniofacial dysmorphism which, if consistent with the entire clinical picture, allows for verification of the diagnosis. It refers especially to PMM2-CDG, manifesting with microcephaly, prominent forehead, flat nasal bridge, thin upper lip and large ears [31]. Recognizable facial features have been described in a number of other CDG [32-33].

The clinical outcome of PMM2-CDG varies among patients [25]. Inverted nipples and abnormal fat distribution were reported as its characteristic features and were present in the majority of our patients. Pericardial effusion was noted in about 30% of PMM2-CDG patients [34]; in our cohort 50% of PMM2-CDG patients. Cardiac involvement in PMM2-CDG seems to be clinically relevant [34]; in our group five PMM2-CDG patients had cardiomyopathy, while it was a rare symptom in non-PMM2-CDG patients.

Abnormal thyroid function was reported in approximately 75% of PMM2-CDG patients [33]; we observed it with a comparable frequency. It results from an abnormal glycosylation of thyrotropine and thyroid-binding globulin [35].

Renal involvement was presented in 17% of PMM2-CDG patients from our cohort vs 6% of PMM2-CDG patients reported in the literature [36]. Our patients had proteinuria, and one of them congenital nephrotic syndrome. Like in the literature, mild proteinuria is the most common renal abnormality in those patients [36].

Some CDG has the peculiar phenotypes being a combination of features. In our cohort, MPI-CDG manifested as purely hepatic (hepatomegaly, elevated liver transaminases) or hepato-intestinal (symptoms as above with recurrent diarrhoea) disease. In SRD5A3-CDG patients, cerebellar ataxia and signs of visual impairment and variable eye malformations, including optic disc hypoplasia, nystagmus, were the characteristic features. ATP6V0A2-CDG, as well as COG7-CDG, were described as cutis laxa syndrome with defective protein glycosylation [37-38]. About 50% of patients with ATP6V0A2-CDG showed cortical malformations, especially pachygyria, in brain MRI. Our patient with ATP6V0A2-CDG showed cutis laxa, pachygyria, as well as polymicrogyria, which expands the spectrum of brain MRI features.

Isoelectric focusing (IEF) of serum transferrin (Tf) is still the method of choice for the diagnosis of N-glycosylation disorders associated with sialic acid deficiency. Our study showed for the first time that ALG13-CDG Tf IEF was abnormal in two our patients, while in ALG1-CDG one patient had normal Tf IEF. There were no reports in the literature. In every patient with clinical and biochemical diagnosis of CDG, molecular analysis has to be performed. It is essential to confirm the diagnosis and also to predict the possible genotype-phenotype correlation. In our study, WES analysis enabled the diagnosis of ALG13-CDG in patients with early-onset drug-resistant epileptic encephalopathy.

The most common pathogenic variant in the *PMM2* gene was p.V231M and p.R141H. The latter is also the most commonly reported in the literature [38]. The compound heterozygotes for p.R141H noted in our study and in the literature as well, were associated with a mild phenotype.

Two deceased patients with ALG1-CDG were compound heterozygous for the p.Ser258Leu. This variant, if present in the homozygous state, is regarded to be related with an early fatal outcome.

Three out of four patients with SRD5A3-CDG presented with an improvement of motor skills and speech development, despite genetic alteration known to results in poor prognosis - c.292_293del, p.Leu98ValfsX121 (in two of them).

The data regarding follow-up of CDG is sparse in the literature. Our *PMM2*-CDG and SRD5A3-CDG patients showed an improvement in motor skills as well as no clinical progression of cerebellar symptoms. In ATP6AP1-CDG some symptoms aggravated with age (from sensorineural hearing loss to total deafness, from hair loss to total alopecia). ALG1-CDG forecast rather a poor outcome with death in the first months of life. Patients with MPI-

CDG on mannose supplementation presented with the improvement of clinical features and Tf isoforms reached values close to reference range. Patient with PGM1-CDG on galactose supplementation had died despite on the improvement of TF isoforms.

5. Conclusions

1. The novel finding was an abnormal Tf IEF pattern in two ALG13-CDG patients and normal in one ALG1-CDG patient.
2. Clinical manifestation of the presented cohort of CDG patients was similar to that reported in the literature.
3. MPI-CDG patients on mannose supplementation presented with the improvement of clinical features and Tf isoforms reached values close to the reference range.
4. PGM1-CDG patient on galactose supplementation had died despite on the improvement of Tf isoforms.
5. Strong, negative linear correlations between asialo-Tf and tetrasialo-Tf, disialo-Tf and tetrasialo-Tf, disialo-Tf and pentasialo-Tf isoforms were detected. Strong, positive linear correlation was found between pentasialo-Tf and tetrasialo-Tf isoforms.
6. PMM2-CDG and non-PMM2-CDG differ significantly in the frequencies of the following symptoms: cerebral ataxia, failure to thrive, hypothyroidism, pericardial effusion, cardiomyopathy, inverted nipples, prolonged INR.

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Table 1. Number of patients presenting a given symptom in the two subcohorts of PMM2-CDG patients and non-PMM2-CDG patients. P-value of the chi-squared test for independence of variables “feature/type of CDG” given in last column; symptoms for which the p-value was less than 0.05 marked with colour.

Figure 1. Isoelectrofocusing (IEF) of serum transferrin isoforms (A - pattern for patient with CDG type II, elevated monosialo-, disjalo- and trisjalotransferrin fraction; B - pattern for patient with CDG type I, elevated asjalo- and disjalotransferrin isoform; C - control normal profile; D - pattern for patient with mild CDG type I, slight elevated asjalo- and elevated disjalotransferrin fraction; E - pattern for patient with mixed CDG I/II on galactose supplementation, mild elevated asjalo-, monosjalo- and disjalotransferrin isoforms; F - pattern for patient with mixed CDG I/II before treatment, elevated asjalo-, monosjalo- and disjalotransferrin isoforms).

Figure 2. Tf isoforms percentage for the whole cohort of patients, different types of CDG marked with different colours. Grey shades represent the reference range. For Asialo-Tf and Monosialo-Tf the reference value is 0.

Figure 3. Correlations between chosen Tf isoforms % in the whole cohort of patients. Pearsons linear correlation coefficients r given in the upper right corner of each panel. Different colours used for different CDG variants.

Figure 4. Effect of treatment in PGM1-CDG patient, for whom several measurements of Tf isoforms were available for the period before the start of treatment, and after. Vertical grey line represents the start of treatment, mean of Tf isoforms % given in each panel as m_1 (before) and m_2 (after the start of treatment).

Ethics approval and consent to participate

The study has been approved by an ethics committee of The Children's Memorial Health Institute in Warsaw, Number 23/KBE/2020. Informed consent was obtained from all included patients.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

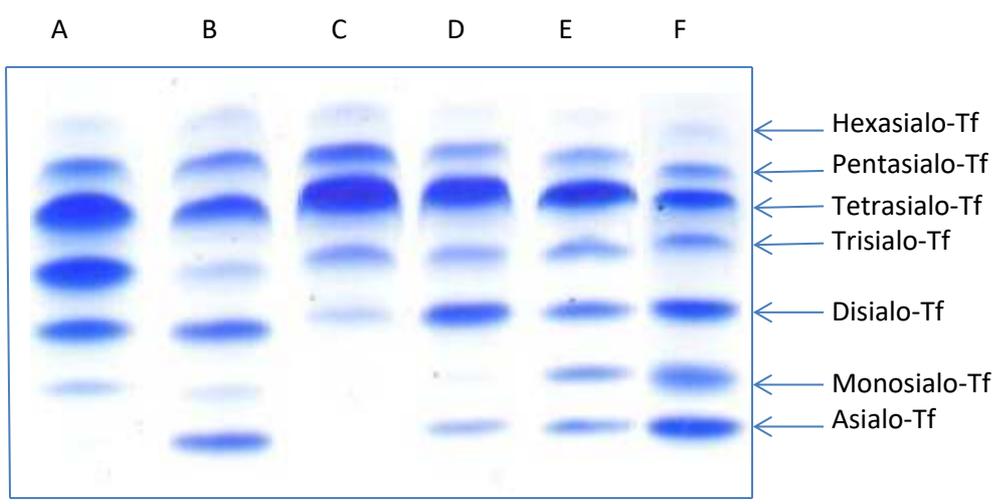
Competing interests

All authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

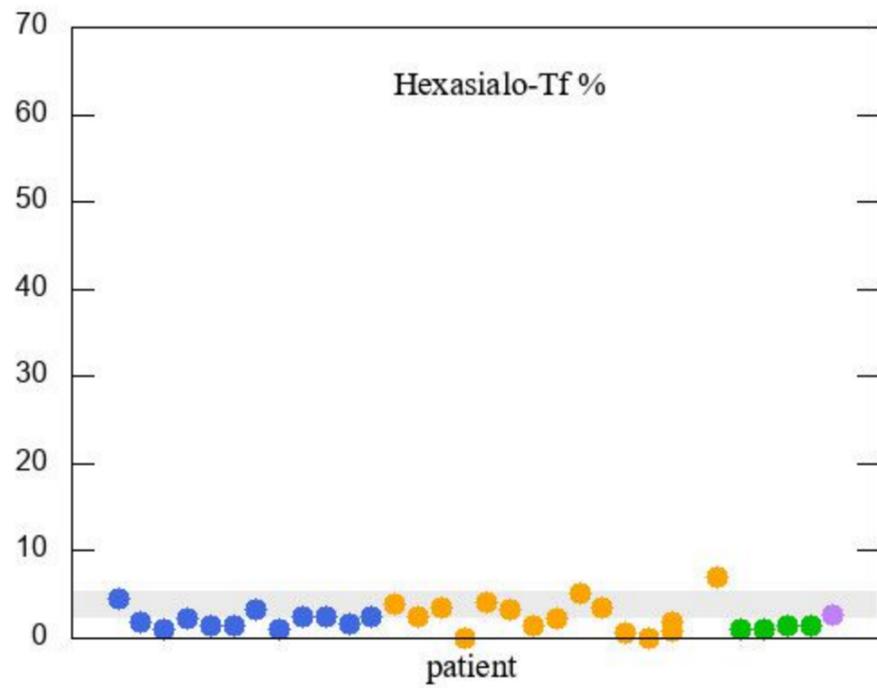
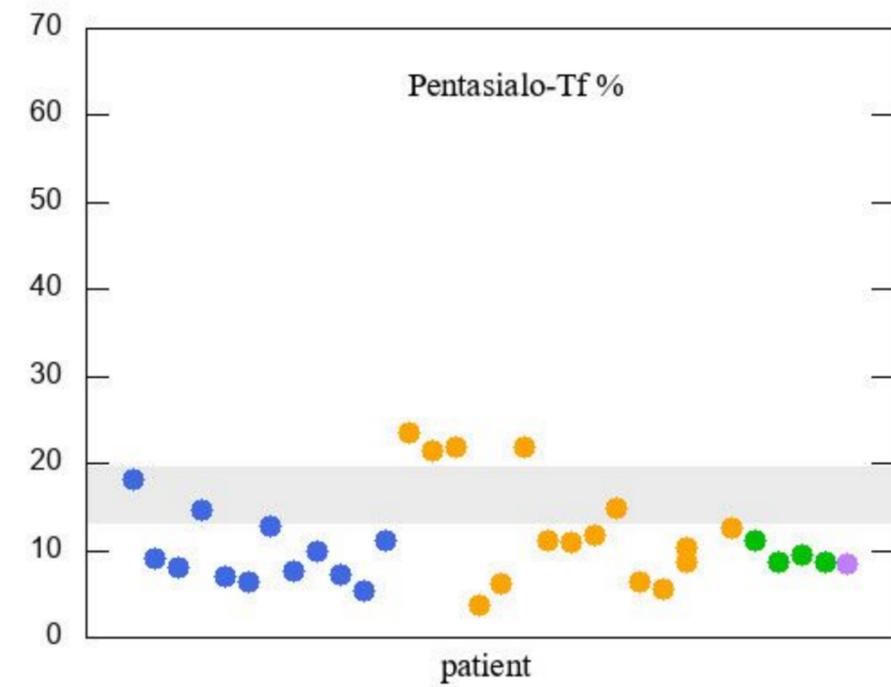
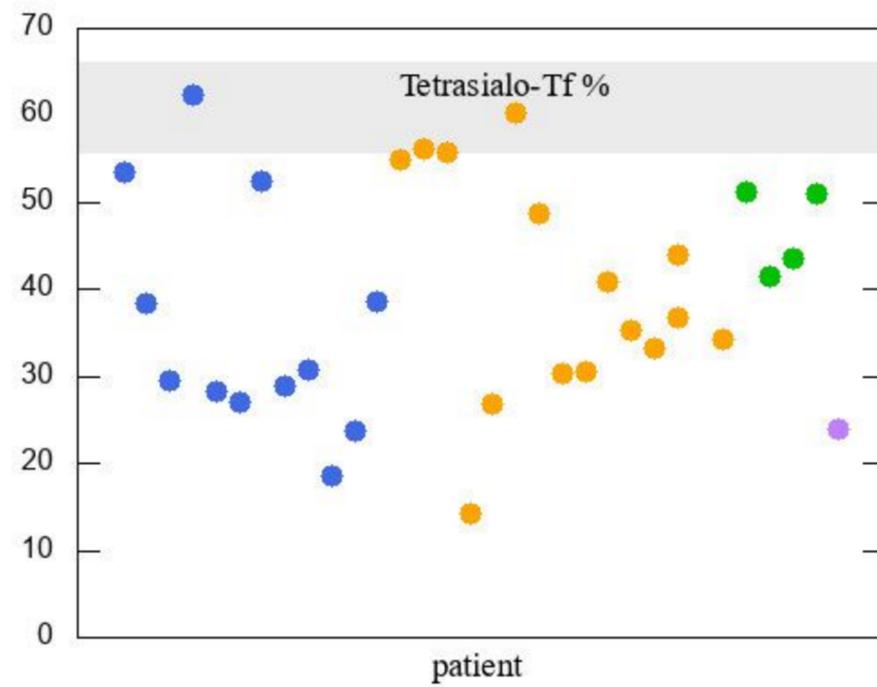
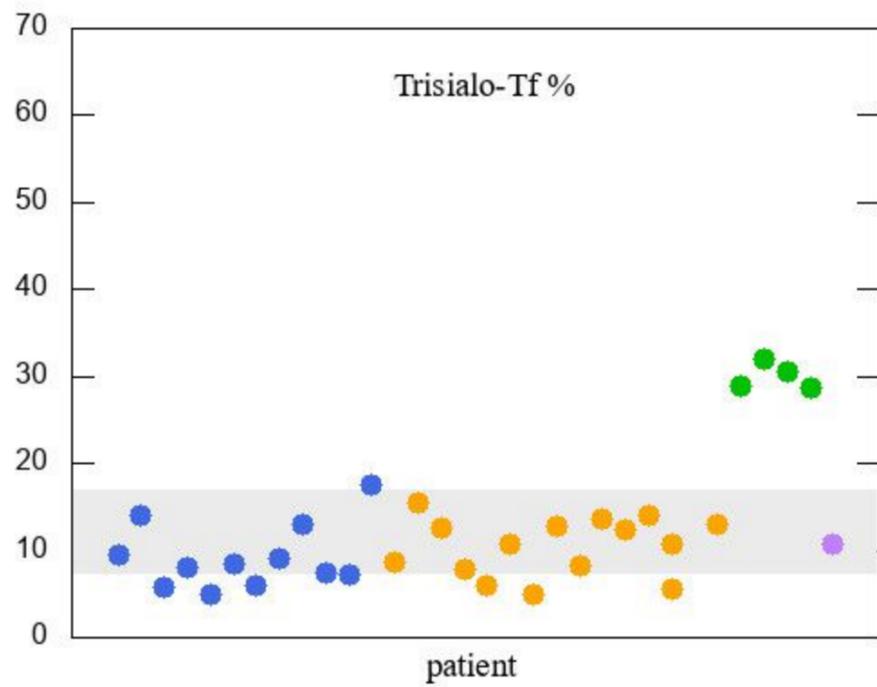
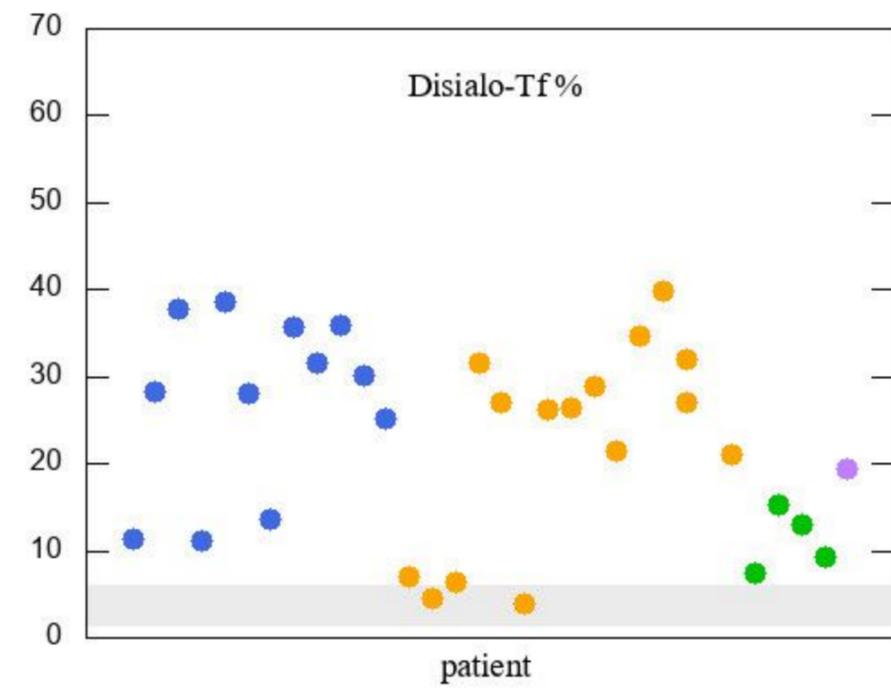
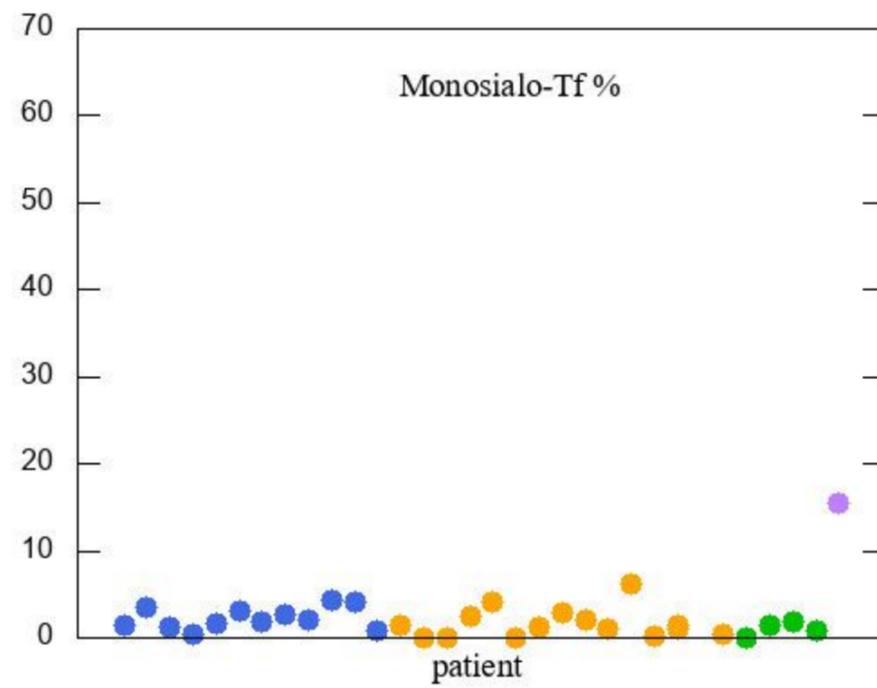
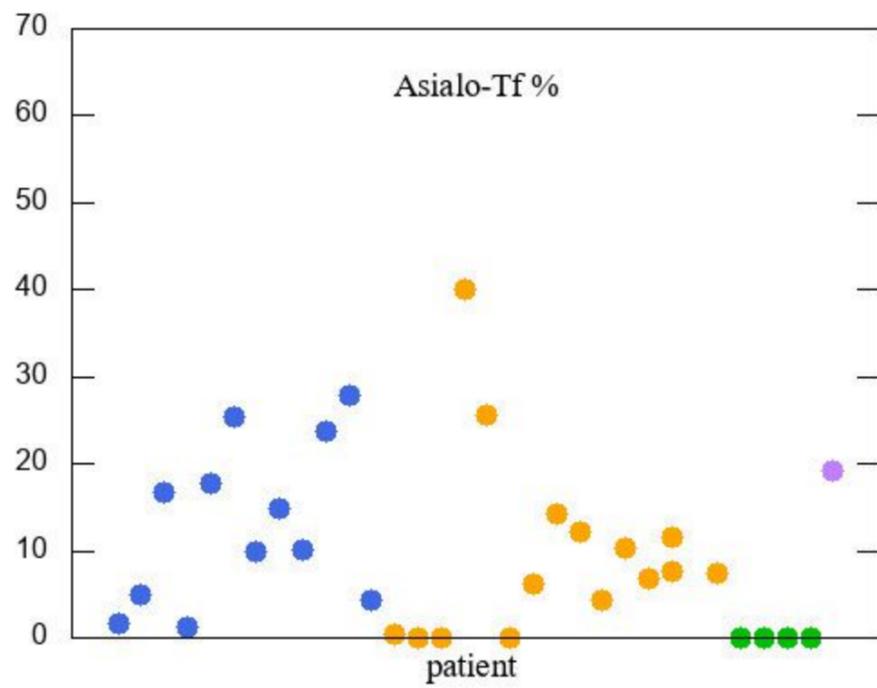
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% Tf-isoforms in the whole cohort

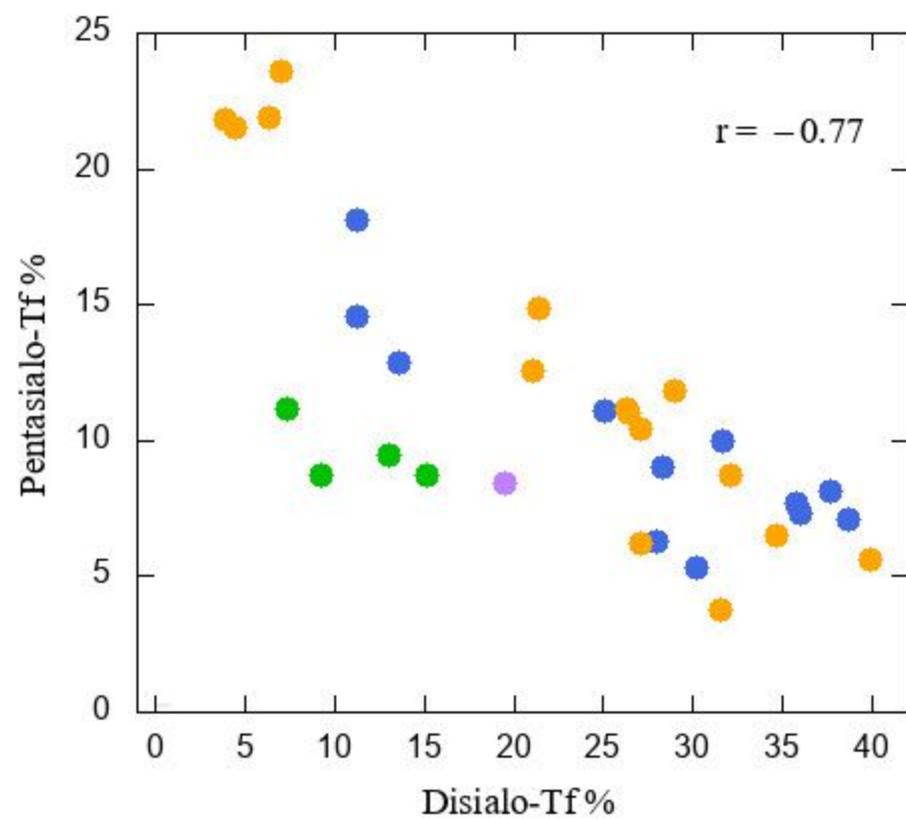
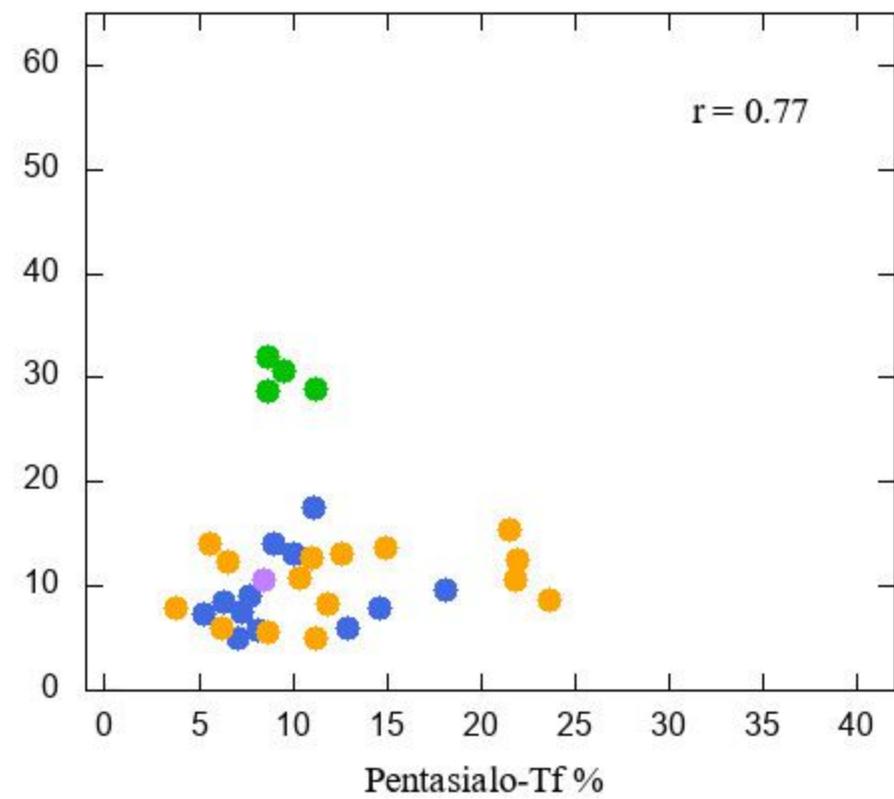
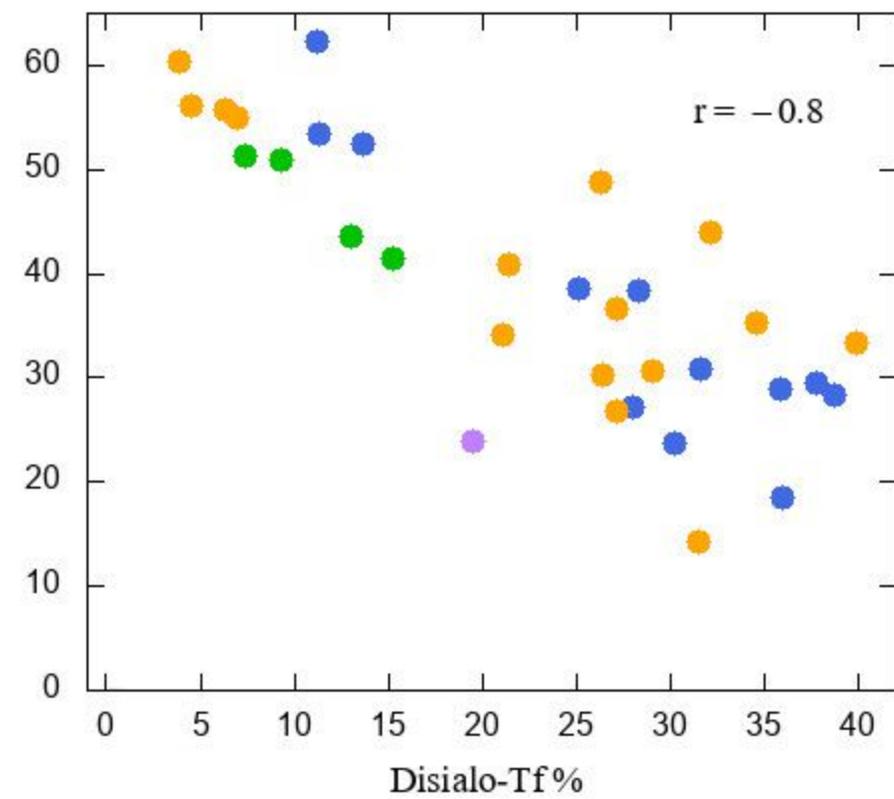
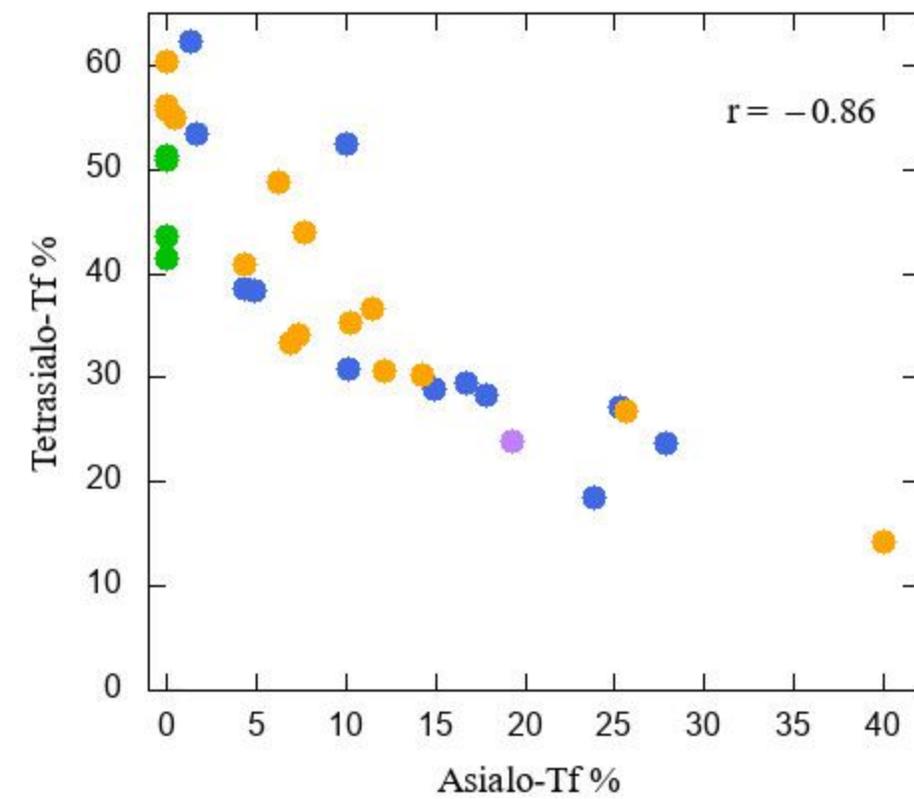


Legend:

- PMM2-CDG
- non-PMM2-CDG-I
- non-PMM2-CDG-II
- non-PMM2-CDG-I/CDG-II

reference range

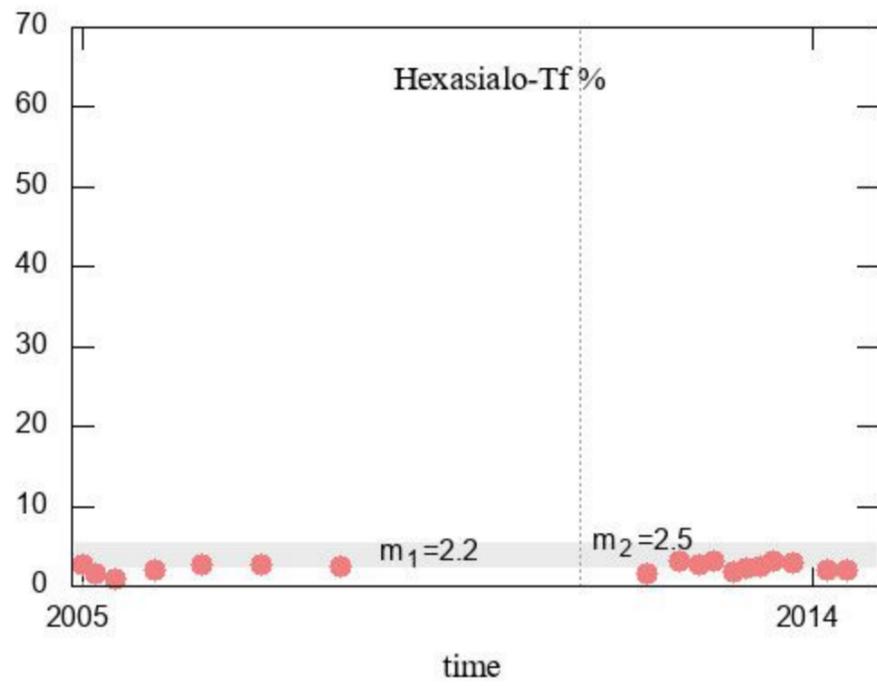
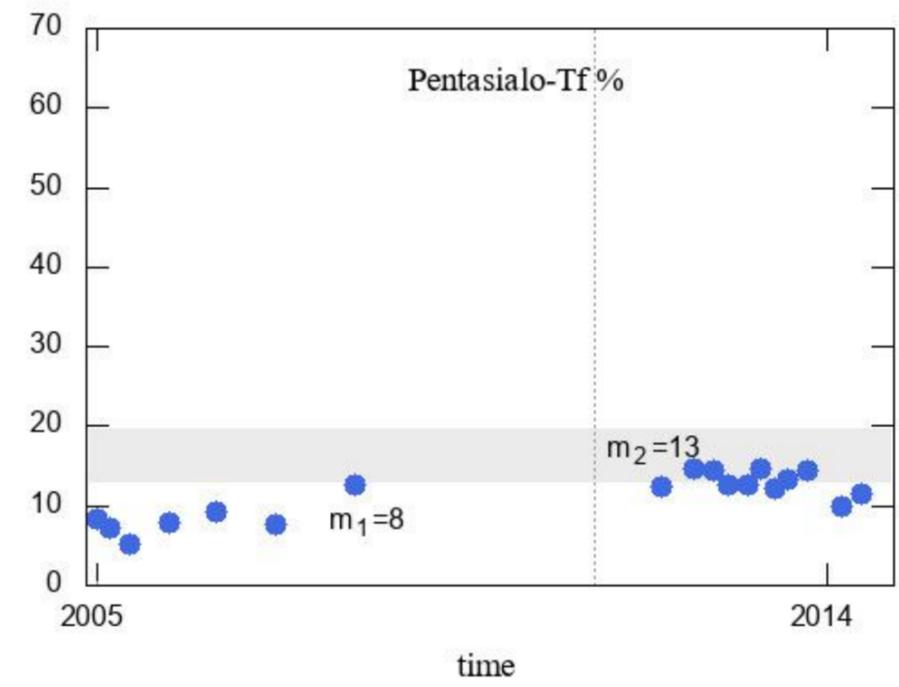
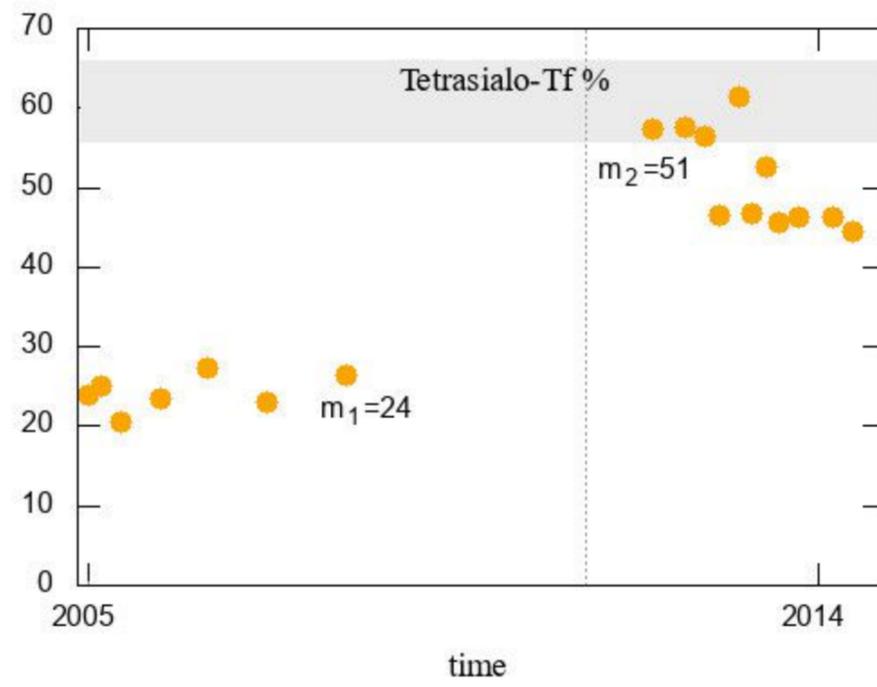
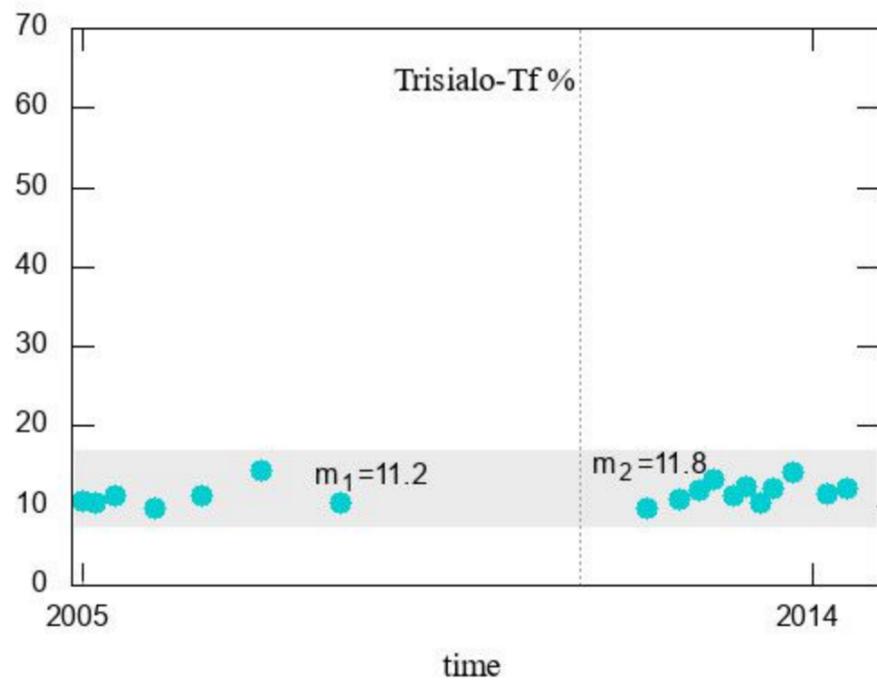
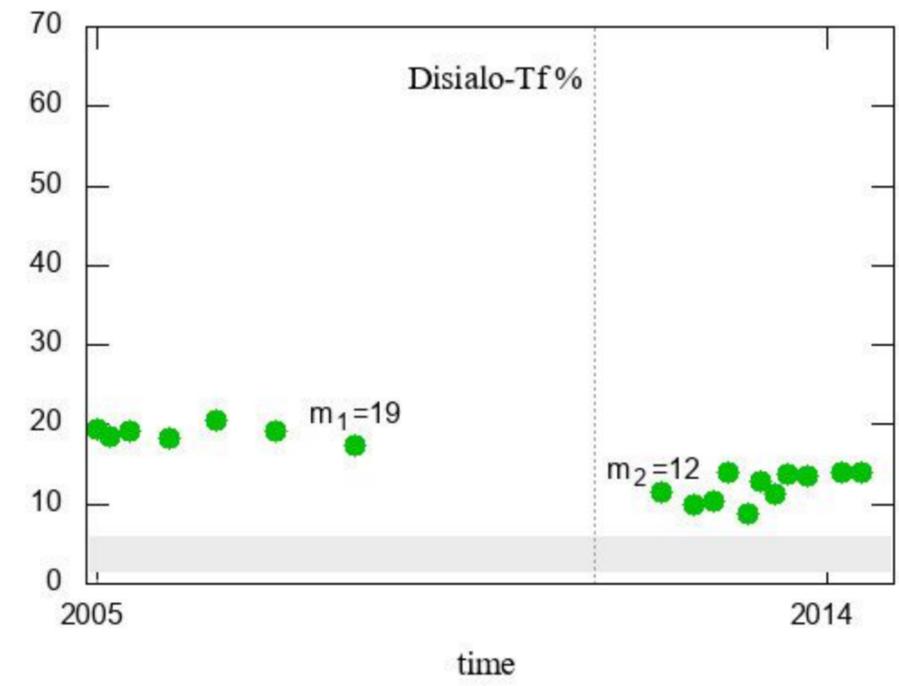
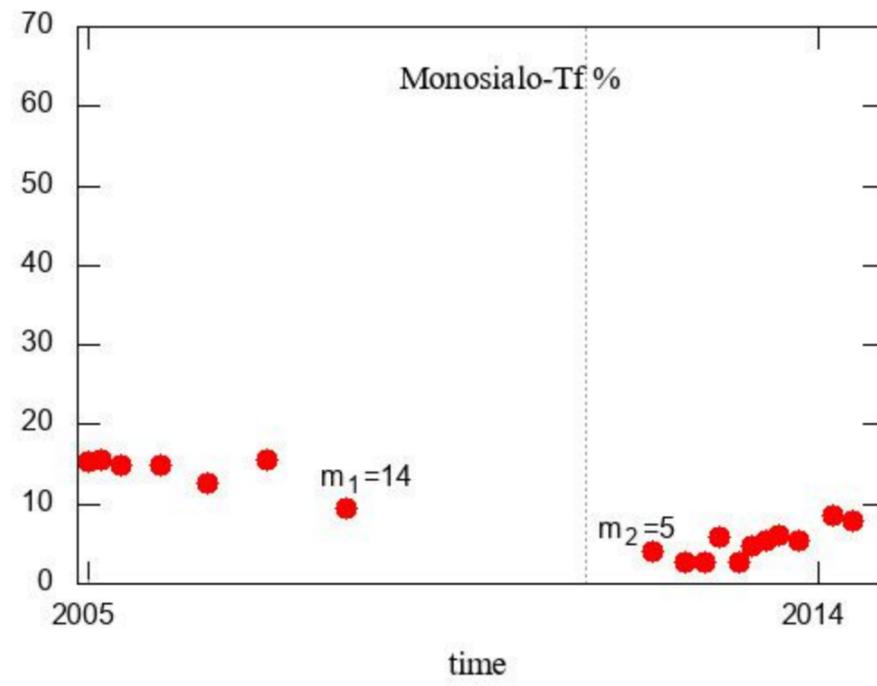
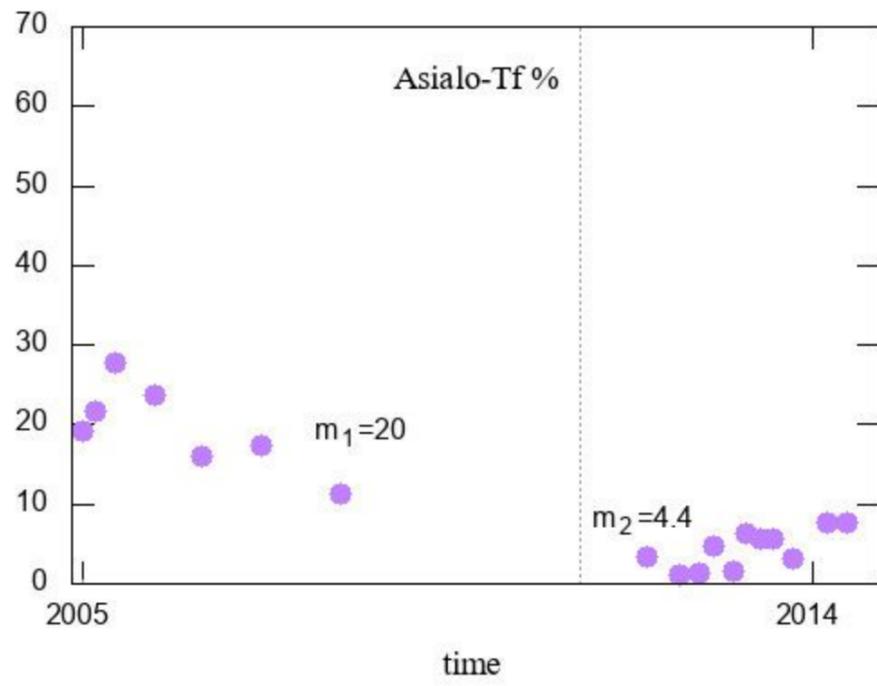
Correlations



Legend:

- PMM2-CDG
- non-PMM2-CDG-I
- non-PMM2-CDG-II
- non-PMM2-CDG-I/CDG-II

PGM1-CDG Patient



Legend:

m_1 - mean of measurements before start of treatment

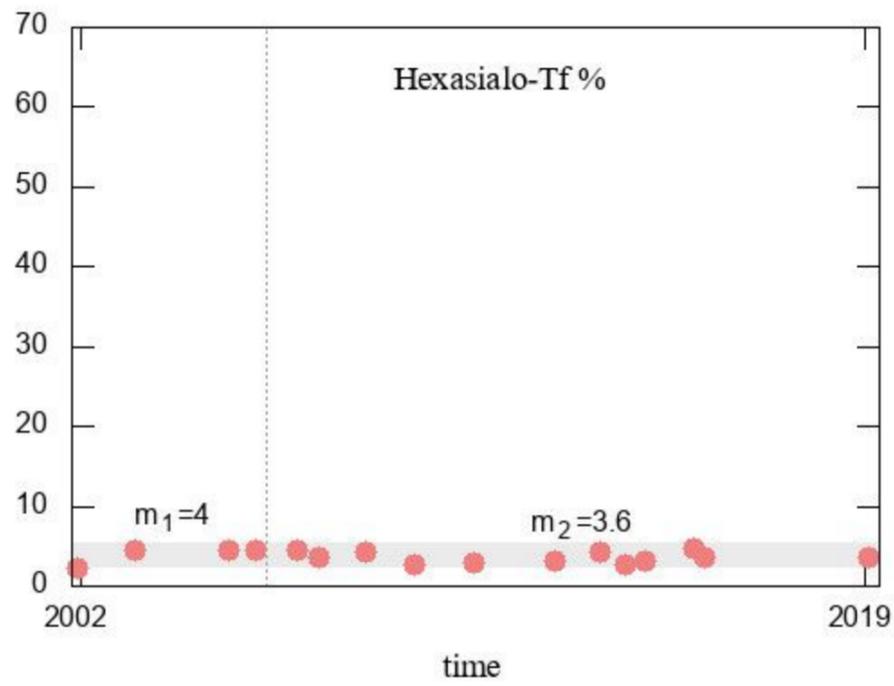
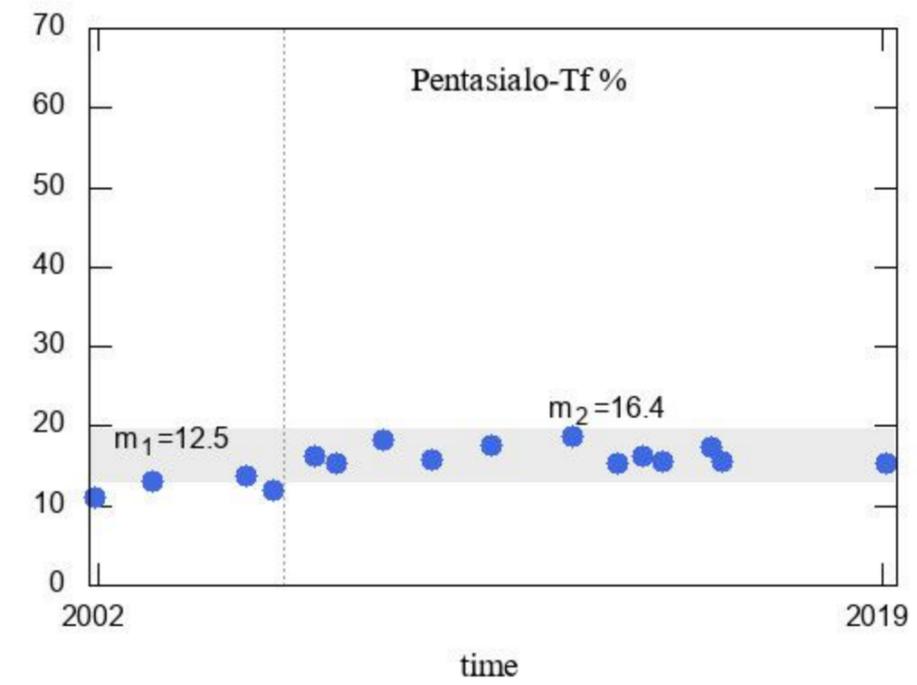
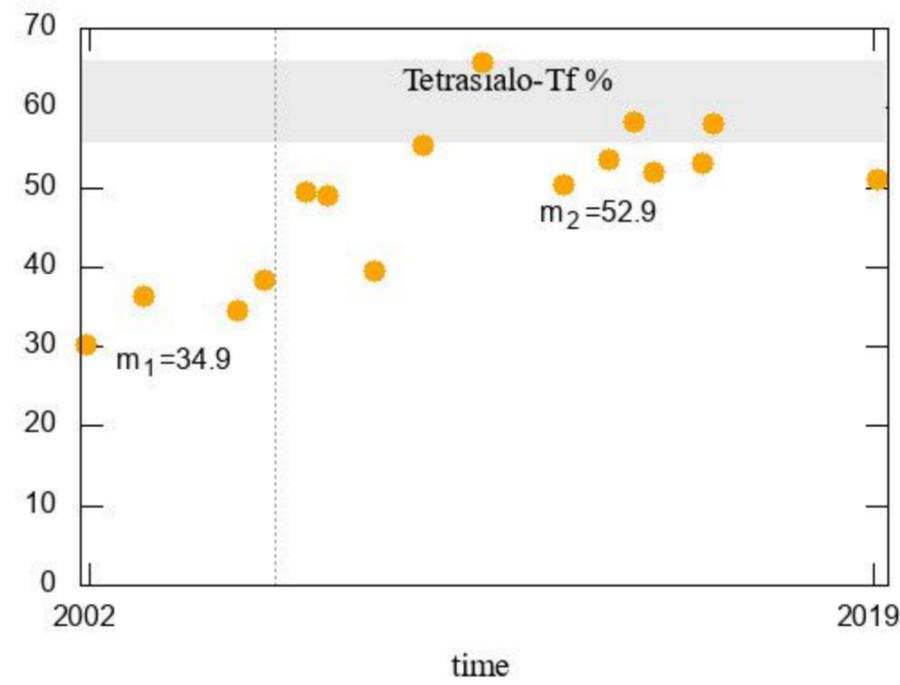
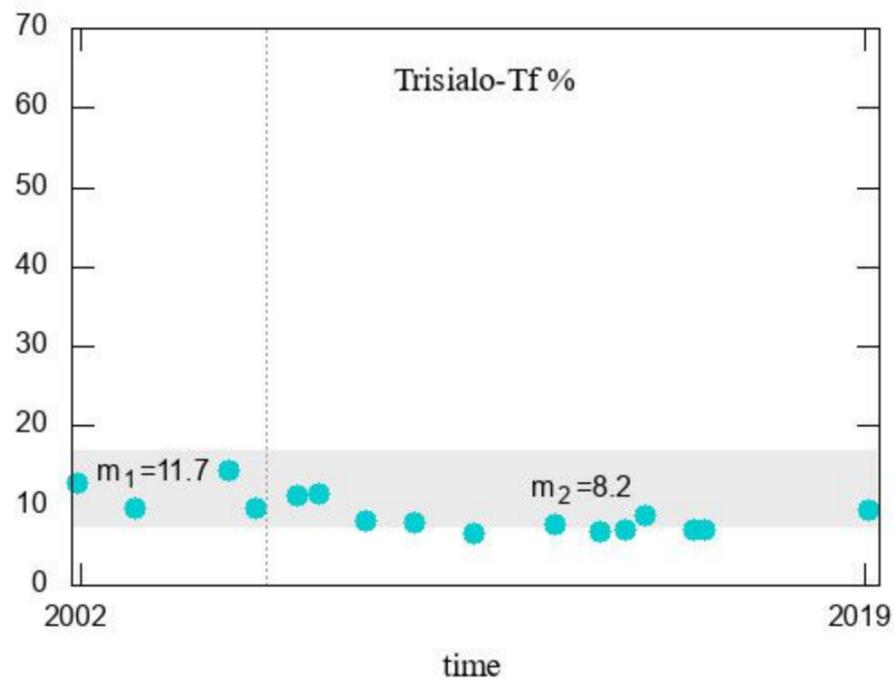
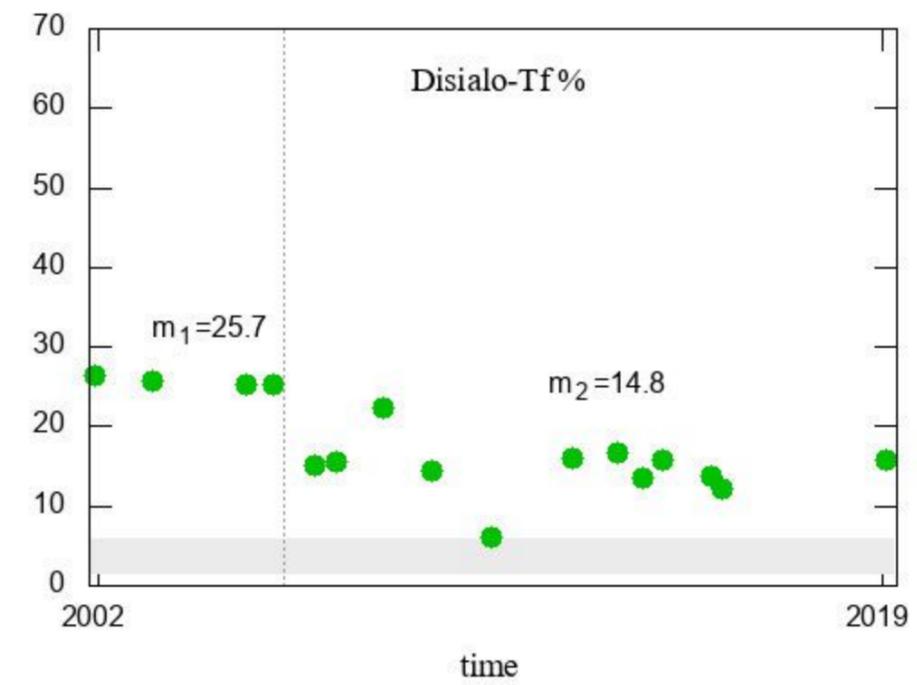
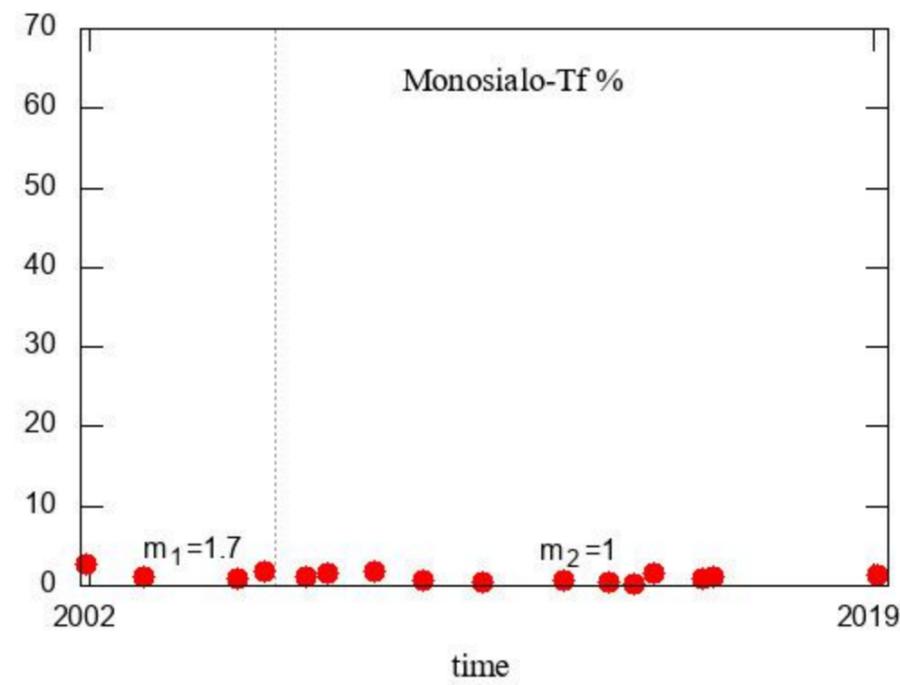
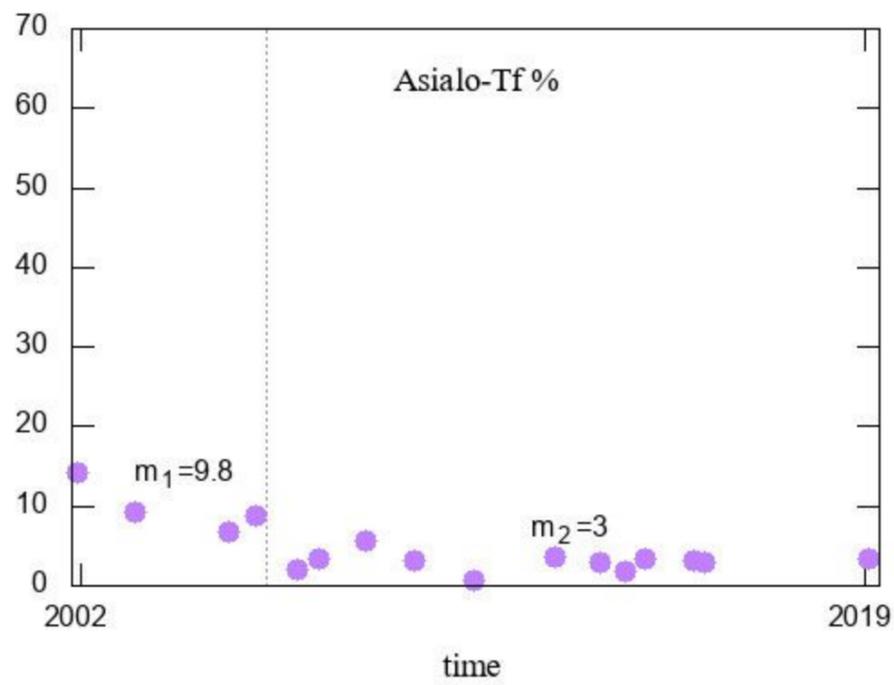
m_2 - mean of measurements after start of treatment

reference range

- start of treatment

Feature	PMM2- CDG	Non-PMM2- CDG	p-value of the chi- squared test for independence
Number of patients	12	20	
Muscle hypotonia	10/12	9/20	0.08
Motor retardation	12/12	13/20	0.06
Cerebellar ataxia	10/10	4/20	0.0002
Seizures	3/12	5/20	1
Microcephaly	2/12	6/20	0.67
Visual impairment	8/12	6/20	0.1
Strabismus	4/12	1/20	0.1
Nystagmus	1/12	4/20	0.71
Optic nerve hypoplasia/atrophy	0/12	1/20	1
Recurrent vomiting/diarrhea	0/12	1/20	1
Hepatomegaly	8/12	6/20	0.1
Failure to thrive	9/12	6/20	0.04
Hypothyroidism	8/10	0/20	<0.0001
Proteinuria	2/12	2/20	1
Pericardial effusion	6/12	1/20	0.01
Cardiomyopathy	5/12	1/20	0.04
Inverted nipples	8/12	0/20	0.0001
Cutis laxa	0/12	1/20	1
Prolonged INR	6/12	2/20	0.04
Elevated serum transaminases	8/12	13/20	1
Low antithrombin III, protein C, and S	6/9	12/15	0.81

MPI-CDG Patient



Legend:

m_1 - mean of measurements before start of treatment

m_2 - mean of measurements after start of treatment

reference range

- start of treatment

Patient	1	2	3	4	5	6	7	8	9	10	11	12
Gender	M	M	M	M	M	M	F	F	M	M	F	M
Age of onset	6m	3m	2m	Neonatal period	4m	Prenatally (non-immune hydrops fetalis)	Neonatal period	Neonatal period	Neonatal period	Prenatally (non-immune hydrops fetalis)	Neonatal period	Neonatal period
Age at diagnosis	6m	3m	5m	6m	6m	1m	1m	6m	2m	2m	2m	1m
Diagnosis	PMM2	PMM2	PMM2	PMM2	PMM2	PMM2	PMM2	PMM2	PMM2	PMM2	PMM2	
Molecular analysis	c.155T>G, p.V52G/ c.640-23A>G, p?	c.422G>A, p.R141H/ c.691G>A, p.V231M	c.169G>A, p.G57R/ c.422G>A, p.R141H	c.422G>A, p.R141H/ c.484C>T, p.R162W	c.357C>A, p.F119L/ c.422G>A, p.R141H	c.24delC, p.C9AfsX27/ c.385G>A, p.V129M	c.24delC, p.C9AfsX27/ c.691G>A, p.V231M	n.a.	c.691G>A, p.V231M/ c.640-15479C>T	c.422G>A, p.R141H/ c.691G>A, p.V231M	c.710C>G, p.T237R/ c.691G>A, p.V231M	c.338C>T, p.P113L; c.470T>C, p.F157S
Age at last follow-up	8y	16y	4y5m	17y	6y	9m	2m	n.a.	12y	2y	9m	18y
Outcome	Alive	Alive	Alive	Alive	Alive	Died	Died	n.a.	Alive	Alive	Alive	Alive
Presentation at diagnosis												
Neurological												
Muscle hypotonia	+	+	+	+	-	+	-	+	+	+	+	+
Motor retardation	+	+	+	+	+	+	+	+	+	+	+	+
Cerebellar ataxia with cerebellar hypoplasia in brain MRI	+	+	n.a.	+	+	+	+	n.a.	+	+	+	+
Seizures	+	+	-	-	-	-	-	-	+	-	-	-
Microcephaly	-	-	+	-	-	-	-	-	+	-	-	-
Hearing impairment	-	+	-	-	-	-	-	-	+	-	-	-
Ocular manifestations												
Visual impairment	+	+	+		+	+	+	-	-	+	-	+
Strabismus	-	-	+	+	+	-	+	-	-	-	-	-
Nystagmus	-	-	-	-	+	-	-	-	-	-	-	-
Gastrointestinal												
Hepatomegaly	-	+	+	-	+	+	+	-	+	+	+	-
Failure to thrive	-	-	+	+	+	+	+	+	+	+	+	-
Endocrine features	-	+	+	-	+	+	+	n.a.	n.a.	+	+	
Renal features												
Proteinuria	-	+	-	-	-	-	-	-	-	-	+	-
Tubulopathy	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac features												
Pericardial effusion	-	-	-	-	+	+	-	+	+	+	+	-
Cardiomyopathy	-	-	hypertrophic	-	-	hypertrophic	+	hypertrophic	+	-	-	-

Skin features	-	-	-	-	-	-	-	-	-	-	-	-
Inverted nipples	-	-	+	+	+	+	-	+	+	+	+	-
Abnormal fat distribution					+	+				+	+	-
Laboratory results												
Prolonged INR	-	-	+	-	-	-	+	-	+	+	+	-
Elevated serum transaminases	-	+	+	-	+	+	+	-	+	+	+	-
Low protein C	-	n.a.	+	-	-	n.a.	+	-	+	n.a.	+	-
Low protein S	-	n.a.	+	-	+	n.a.	+	-	+	n.a.	+	-
Low anithrombin	-	n.a.	+	-	n.a.	n.a.	+	-	+	+	+	-
Follow-up												
	Improvement of motor skills, epilepsy well controlled by medication, normal liver volume, normal serum transaminases	Improvement of motor skills, epilepsy controlled by medication, Proteinuria since 8m, renal biopsy revealed the congenital nephrotic syndrome	Failure to thrive, subtle improvement of motor skills	Intellectual disability from mild (7y) to moderate (17y), epilepsy since 15y, well controlled by medication	Improvement of motor skills, stroke-like episode at 6y, Normal serum transaminaes	Pancytopenia, progressive course	Mechanical ventilation since 2nd week of life; Post mortem examination – liver fibrosis with cholestasis, hypertrophy of the left ventricle	No follow-up	Thrombotic event at 2m, pleural-pericardial window at 9m, Epileptic seizures from 10m, Drug-resistant epilepsy diagnosed at 12y	Improvement of motor skills, Feeding by nasogastric tube, enlarged liver volume, Elevated serum transaminases	Feeding by nasogastric tube, several episodes of pericardiocentesis, enlarged liver volume, elevated serum transaminases, coagulopathy, thrombocytopenia, proteinuria	Epilepsy since 2y, peripheral neuropathy, profound intellectual disability (17y), wheel-chair dependent

Laboratory results											
Prolonged INR	-	-	-	+	-	-	-	-	-	-	-
Elevated serum transaminases	-	-	-	+	-	-	-	+	+	+	+
Low protein C	-	-	-	+	+	+	+	+	+	+	n.a.
Low protein S	-	-	-	+	+	+	+	+	+	+	n.a.
Low anithrombin	-	-	-	+	+	+	+	+	+	+	+
Follow-up											
	Drug-resistant epilepsy, Severe mental retardation (4y), subtle improvement of motor skills, walks with help (4y), microcephaly	Drug-resistant epilepsy, improvement of motor skills, walks independently (2y), speaks few words, microcephaly	Drug-resistant epilepsy	Spastic tetraparesis, two trombotic events, profound deficiency of antithrombin, protein C and S	Mechanicalm ventillation since 4m of age	Failure to thrive, microcephaly, profound hypotonia	Hypotonia, cachexia, brain atrophy on brain MRI, normal serum transaminases	Mannose treatment since diagnosis, at 21y presenting with normal intellectual development, normal liver volume, normal serum transaminases	Mannose treatment since diagnosis, at 14y 5m presenting with normal intellectual development, normal liver volume, normal serum transaminases	Mannose treatment since diagnosis, at 4y presenting with normal psychomotor development, normal liver volume, normal serum transaminases, and no diarrhea	Progressive cardiac insufficiency since 4y, dilated cardiomyopathy diagnosed at 9y, elevated serum transaminases since 4y, liver biopsy at 10y with results of liver steatosis, start treatment from 16y

Cardiac features									
Pericardial effusion	-	-	-	-	-	-	-	-	-
Cardiomyopathy	-	-	-	-	-	-	-	-	-
Skin features	-	-	-	-	-	cutis laxa	-	-	-
Laboratory results									
Prolonged INR	-	-	-	+	-	-	-	-	-
Elevated serum transaminases	+	+	+	+	+	-	+	+	+
Low protein C	+	+	+	+	n.a.	+	n.a.	n.a.	n.a.
Low protein S	+	+	+	+	n.a.	+	n.a.	n.a.	n.a.
Low anithrombin	+	+	+	+	n.a.	+	n.a.	n.a.	n.a.
Follow-up									
	Improvement in motor skills, walks independently, Normal serum transaminases	Improvement in motor skills, walks independently but abnormal gait pattern (ataxia), says few words, normal serum transaminases	Feeding by gastrostomy (implemented at 6m), severe intellectual disability (9y), tetraparesis	Says few words at 8-9y and few sentences at 10-11y Moderate intellectual disability (11y), walks independently from 9y of age but abnormal gait pattern (ataxia), Normal serum transaminases	Mechanical ventilation since 6m	No follow-up	Sensorineural hearing loss (since 8y) requiring cochlear implants, progressive hair loss since 15y, total alopecia and dark (chestnut) skin at 25y, Normal liver volume, normal serum transaminases, normal urine analysis	Sensorineural hearing loss (since 6y) requiring cochlear implants, at 18y of age presenting with progressive hair loss, leukopenia, mild elevation of serum transaminases, hypogammaglobulinemia, and glomerular proteinuria	Sensorineural hearing loss from childhood requiring cochlear implants, at 36y of age presenting with total alopecia, dark (chestnut) skin, leukopenia, mild elevation of serum transaminases, and glomerular proteinuria

Table Z

A	Asiao-	Monosialo-	Disialo-	Trisialo-	Tetrasialo-	Pentasio-	Heksasio-
Asiao-	1	0,447847	0,640871	-0,52112	-0,85529	-0,65419	-0,20206
Monosialo-		1	0,230483	-0,16668	-0,49676	-0,37054	-0,04772
Disialo-			1	-0,4376	-0,80482	-0,7663	-0,36403
Trisialo-				1	0,262683	0,008233	-0,17141
Tetrasialo-					1	0,77847	0,198376
Pentasio-						1	0,536565
Hexasio-							1

B	Asiao-	Monosialo-	Disialo-	Trisialo-	Tetrasialo-	Pentasio-	Heksasio-
Asiao-	1	0,672755	0,642791	-0,49707	-0,82465	-0,83963	-0,53543
Monosialo-		1	0,425794	-0,09801	-0,65875	-0,63311	-0,23217
Disialo-			1	-0,10587	-0,91137	-0,8544	-0,7296
Trisialo-				1	0,087538	0,177861	0,181705
Tetrasialo-					1	0,89102	0,583147
Pentasio-						1	0,824373
Hexasio-							1

C	Asiao-	Monosialo-	Disialo-	Trisialo-	Tetrasialo-	Pentasio-	Heksasio-
Asiao-	1	0,526325	0,554648	-0,39629	-0,87679	-0,73787	-0,26578
Monosialo-		1	0,504972	-0,22847	-0,54566	-0,56565	-0,20682
Disialo-			1	-0,24566	-0,77892	-0,95091	-0,45461
Trisialo-				1	0,16167	0,295465	0,095761
Tetrasialo-					1	0,861673	0,162902
Pentasio-						1	0,449505
Hexasio-							1

Supplementary Table S2. Pearson's correlation coefficients for % isoforms for the whole cohort of patients (A), PMM2-CDG patients (B), and non-PMM2-CDG patients (C). Correlation values $|r| > 0.7$ marked with colour, the intensity of the hue reflecting the strength of the correlation.

Figures

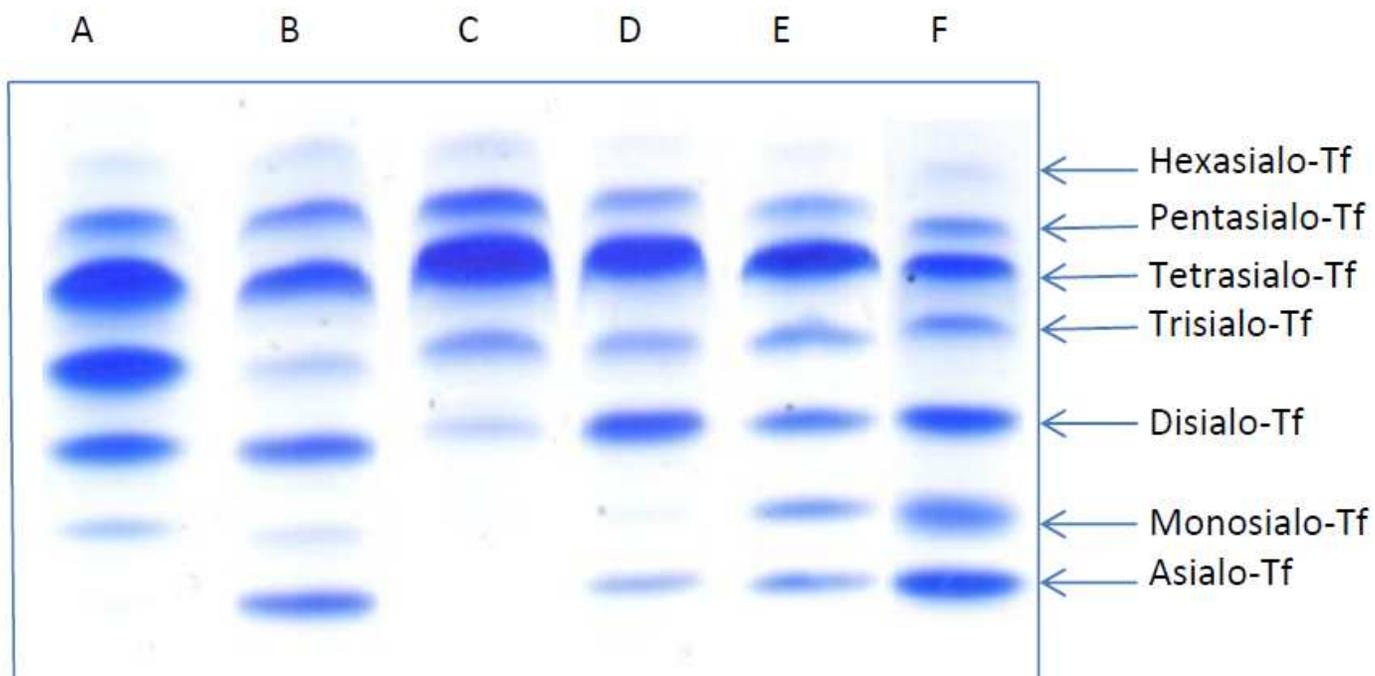


Figure 1

Isoelectrofocusing (IEF) of serum transferrin isoforms (A - pattern for patient with CDG type II, elevated monosialo-, disialo- and trisialotransferrin fraction; B - pattern for patient with CDG type I, elevated asialo- and disialotransferrin isoform; C - control normal profile; D - pattern for patient with mild CDG type I, slight elevated asialo- and elevated disialotransferrin fraction; E - pattern for patient with mixed CDG I/II on galactose supplementation, mild elevated asialo-, monosialo- and disialotransferrin isoforms; F - pattern for patient with mixed CDG I/II before treatment, elevated asialo-, monosialo- and disialotransferrin isoforms).

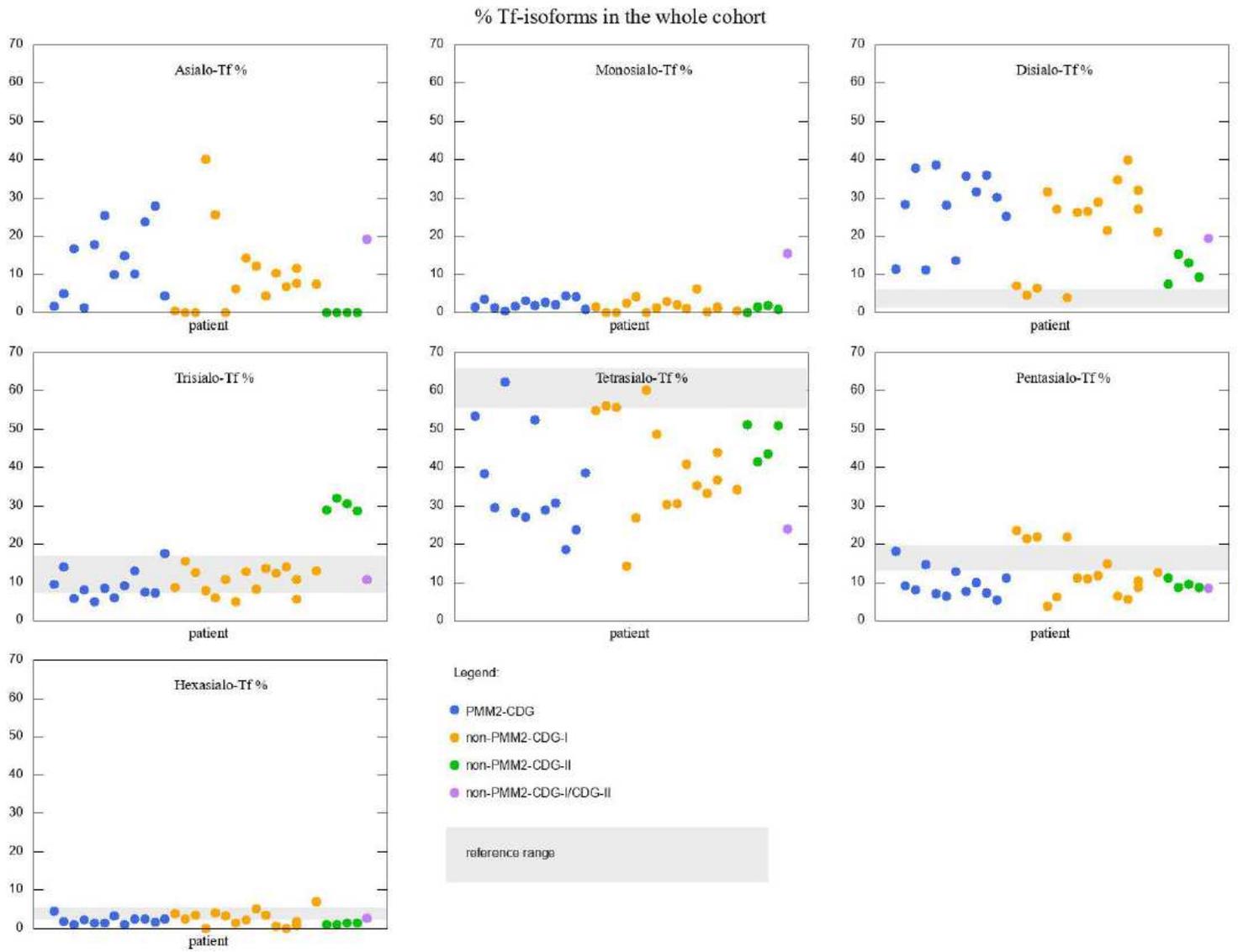


Figure 2

Tf isoforms percentage for the whole cohort of patients, different types of CDG marked with different colours. Grey shades represent the reference range. For Asialo-Tf and Monosialo-Tf the reference value is 0.

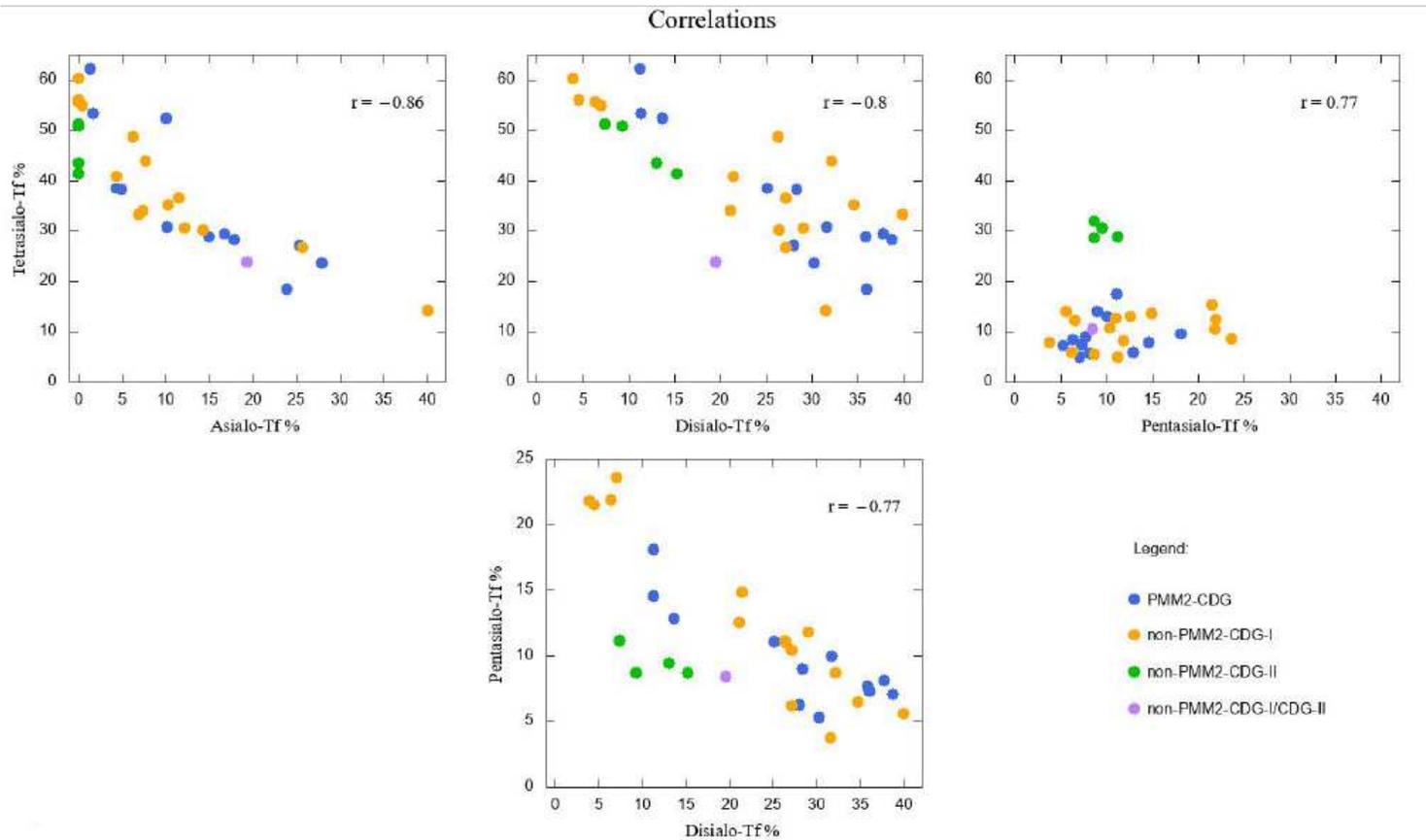


Figure 3

Correlations between chosen Tf isoforms % in the whole cohort of patients. Pearson's linear correlation coefficients r given in the upper right corner of each panel. Different colours used for different CDG variants.

PGM1-CDG Patient

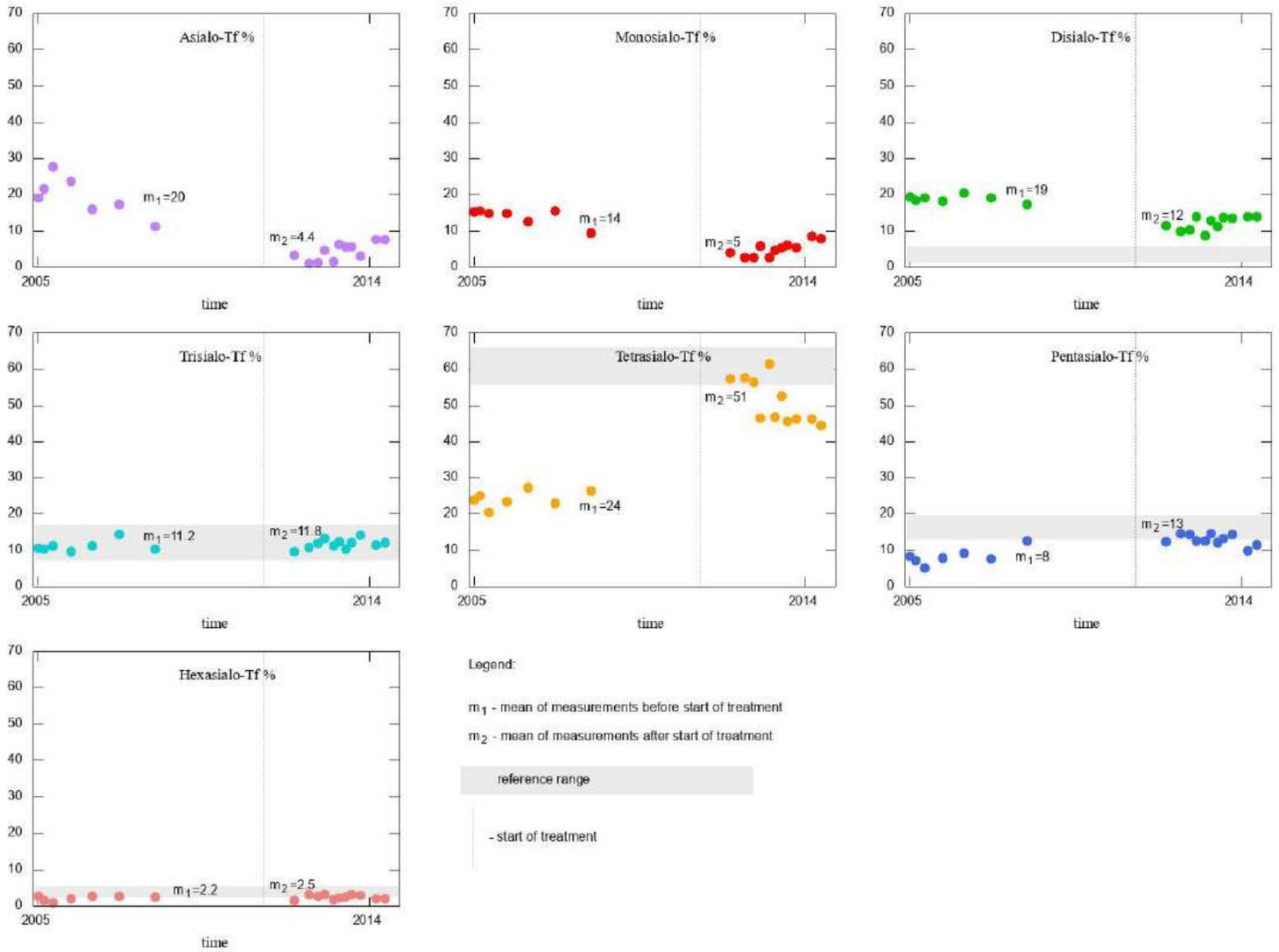


Figure 4

Effect of treatment in PGM1-CDG patient, for whom several measurements of T_f isoforms were available for the period before the start of treatment, and after. Vertical grey line represents the start of treatment, mean of T_f isoforms % given in each panel as m₁ (before) and m₂ (after the start of treatment).