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CAT
Critically Appraised Topic

Coeliac disease: kinetics of antibody titers and clinical correlation after initiation of gluten-free diet

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CLINICAL BOTTOM LINE

Serologic assays are well established tools for the diagnosis of coeliac disease (CD), but their usefulness to monitor CD is less evidenced-based. In this prospective study, 13 newly diagnosed CD patients were followed for 2 years. They consulted the gastro-enterologist (or paediatrician) 0, 3, 6, 12 and 24 months after start of the gluten-free diet (GFD) and filled out a complaint diary questioning various (extra-)intestinal symptoms. A blood sample was drawn to analyze serological and routine biochemical/haematological parameters. Eight CD serological assays were performed to evaluate the kinetics of the antibody (Ab) titers: tissue transglutaminase (tTG) IgA/IgG and deamidated gliadin peptides (DGP) IgA/IgG from two manufacturers (Thermo Fisher and INOVA).

The results obtained with the serological assays of the two manufacturers were similar. Fully - and partially compliant patients showed a rapid decline in all Ab titers. The strongest decline was observed for tTG IgA in completely histologically recovered patients, the weakest for tTG IgG due to the low sensitivity of this marker. Non-compliant patients continued to have elevated Ab titers. None of the evaluated serological assays fully correlated with patient's complaints or persisting biochemical/haematological abnormalities. Using the manufacturer's cut-off, a significant difference in clinical interpretation of the tTG IgA assay was revealed between Thermo Fisher and INOVA. By harmonizing cut-offs based on a predefined 100% specificity level, a similar mean time to serological normalization was obtained (11.9 months for fully – and partially compliant patients). Clinicians must be aware of the inter-manufacturer differences and should rely on cut-offs with a pre-defined specificity to harmonize clinical decision making.

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CLINICAL/DIAGNOSTIC SCENARIO

With a prevalence of 0.5-1%, celiac disease (CD) is a common chronic autoimmune-mediated enteropathy triggered by gluten ingestion causing villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis of the small intestine.[1] Furthermore, the prevalence is increasing in areas with high frequency CD-predisposing genes (HLA-DQ2/DQ8) and high gluten consumption.[2] Additional risk factors include female sex (female:male ratio 1.5:1), first-degree CD relatives, patients with Down syndrome, IgA deficiency and other autoimmune disease (*e.g.* type I diabetes).[2,3] The clinical presentation of CD is age-dependant,[1] can vary greatly between patients[2] and is as such classified into 'symptomatic', 'sub-clinical', 'potential' and 'refractory'. Patients who are 'symptomatic' can endure "classic" intestinal symptoms (chronic diarrhoea, bloating, constipation, abdominal pain) or "non-classic" extra-intestinal symptoms (dermatitis herpetiformis, failure to thrive, iron deficiency, chronic fatigue, headache, osteoporosis).[4,5] However, since these "non-classic" symptoms are more common compared to "classic" symptoms, CD is often undiagnosed causing considerably impaired quality of life.[4] In addition, untreated CD (and 'refractory' CD) can induce severe complications as neurologic disorders, infertility, ulcerative jejunoileitis and intestinal lymphoma.[2,3]

Besides clinical symptoms, two key elements are used in clinical practice for diagnosing CD: serological testing and biopsy-based morphological examination of the small intestinal mucosa. Both recent American[6] and European[7,8] guidelines advise the detection of tissue transglutaminase (tTG) and endomysial antibodies (EMA) given their high sensitivity and specificity, but omit the use of anti-gliadin antibodies (AGA) due to lower diagnostic performance.[9–14] At all ages and in both symptomatic and asymptomatic (at-risk) patients consuming a gluten-containing diet, an immunoglobulin (Ig)A tTG assay (using an enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA) or fluorescence enzyme immunoassay (FEIA) technique) is preferred as first-line approach because of its greater reproducibility compared to the labour-intensive and observer-dependent indirect immunofluorescence (IIF)-based IgA EMA test.[15] There are recognized differences in test performance between various commercially available tTG IgA assays,[16] but overall there is consistency in the sensitivity and specificity of the test, both being 95% in untreated CD.[6] Furthermore, the higher the titer of the tTG IgA result, the higher the likelihood ratio (LR) of CD.[17–19] In combination with tTG IgA, total IgA should be measured, as IgA deficiency occurs 10x more frequently in CD patients (2-3%).[8,20] In patients with selective IgA-deficiency (*i.e.* total serum IgA <0.07 g/L), deamidated gliadin peptides (DGP) IgG together with tTG IgG are considered the best tool for identifying CD.[21,22] Combining several tests for CD instead of tTG IgA alone may marginally increase sensitivity but reduces specificity and is therefore not recommended in low risk populations.[23] However, combining double DPG IgG and tTG IgA antibody positivity results in a higher LR of CD, which in specific diagnostic cases can add value in clinical decision making.[24]

Despite a good diagnostic performance of CD serology, current CD guidelines for adults patients still recommend positive serological screening to be followed by duodenal biopsy in low prevalence populations (*i.e.* <5%) to ensure a correct diagnosis before imposing a lifelong GFD to the patient.[6] This histological approach additionally enables the assessment of severity of mucosal damage by modified Marsh classification.[8,20] This classical two-step approach is mainly abandoned in diagnosing childhood CD. The 2012 European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines proposed to omit duodenal biopsy when a patient has tTG IgA levels above 10-fold the upper limit of normal (10 x ULN)[25] and advises in their latest 2020 guidelines to confirm CD in a second blood sample with positive EMA IgA antibodies.[7] This non-biopsy approach may reduce 30-50% of biopsies which is not only an avoidance of procedural risk, but also cost-saving without compromising on specificity.[20,26] Since nominal thresholds for tTG IgA levels are not aligned across assays, clinical interpretation of tTG IgA results improves by defining diagnostic cut-offs based on predefined LRs or specificity.[18,19,24]

A gluten-free diet (GFD) is the only effective treatment for CD as there are currently no medications that can reliably and safely prevent the mucosal damage caused by exposure to gluten.[27] A systematic review supports the role of strict adherence to the GFD to control symptoms, improve quality of life, and decrease the risk of complications.[28] Following a GFD can be cumbersome and strict avoidance of gluten is difficult because there are many hidden sources of gluten in commercial food products. Therefore, long-term multidisciplinary monitoring of CD patients remains essential.[29] Persistent or recurring symptoms should lead to a reassessment of the patient's original diagnosis, a review of the GFD and an evaluation for disorders associated with CD (microscopic colitis, pancreatic exocrine dysfunction), complications of CD or refractory CD.[23]

Currently, the kinetics of CD serology and its role in the monitoring of CD patients is a matter of debate.[6,30,31] Although persistent symptomatology and/or lack of declining serology after 1 year strongly suggest ongoing gluten intake,[8,32,33] a recent meta-analysis showed a fairly low positive predictive value (PPV) and sensitivity of positive tTG IgA titers to detect persistent villous atrophy in patients on a GFD (38% for adults, 70% for children). The negative predictive value (NPV) of positive tTG IgA results to detect ongoing mucosal damage was higher in patients on a GFD (specificity in adults 80%, in children 87%).[34] So it can be concluded that a negative serological result does not provide information to the clinician regarding persisting villous atrophy for which biopsy remains the golden standard. A refinement of the tTG IgA determination utilizing the detectable levels below the upper normal limit may add in the identification of CD patients with mucosal healing.[35]

In this Critically Appraised Topic (CAT), we aimed to investigate the Ab kinetics of eight serological assays (tTG IgA/IgG, DGP IgA/IgG of manufacturers Thermo Fisher (FEIA) and INOVA (CLIA)) in 13 newly diagnosed and well-characterized CD patients. Follow-up visits were conducted after 3 -, 6 -, 12 - and 24 months after CD diagnosis in which there was (1) an assessment of compliance with the GFD, (2) a description a patient complaint dairy and (3) a blood sample drawn to investigate several routine laboratory tests besides the CD serological assays. Differences in Ab titer, complaints and routine laboratory tests between strictly, partially and non-compliant patients were investigated.

QUESTIONS

1) *How does the kinetic profile differ between the different serological assays (tTG IgA/IgG vs. DGP IgA/IgG)?*
2) *Is the clinical interpretation dependent on the assay that is used (Thermo Fisher vs. INOVA)?*
3) *How does serological status, complaints and routine laboratory test differ between strictly, partially and non-compliant CD patients?*

SEARCH TERMS

1) *MeSH Database (PubMed): "celiac disease [MeSH term]", "autoantibodies/immunology [MeSH term]", "diet, gluten free [MeSH term]", "follow up studies [MeSH term]"*
2) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane)*
3) *International organizations: American College of Gastroenterology (ACG; www.gi.org); ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition)*
4) *UpToDate Online*

RELEVANT EVIDENCE/REFERENCES

1) *Guidelines and Recommendations (most recent topics on top)*

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5) Reference Works, Handbooks and Databases
    - Not applicable

6) Posters, "grey literature", presentations
    - Not applicable

1. Material & Methods

1.1 Study population

Newly diagnosed CD patients (>2 years old), confirmed by positive serology and biopsy, presenting at the OLV Hospital Aalst (Belgium) were prospectively included starting at November 2016. By signing informed consent, all patients agreed to consult the gastro-enterologist/paediatrician at t = 0 months (start of GFD), t = 3 months (not routinely performed) and at t = 6, 12 and 24 months (routine performed follow-up visits). At these time points, clinical information was collected, including the assessment of adherence to GFD by the clinician. Patients were asked to fill out a patient complaints diary in which they had to rate ten symptoms from 0 (no complaints) to 10 (many complaints) based on the past days. Following symptoms required assessment: anorexia/refusal to eat, persistent diarrhoea, constipation, loss-of-weight, vomiting, irritability, skin problems, aphtous stomatitis, headache and tiredness. In addition, blood samples were collected at these five time points to assess serological status (see below), haemoglobin [g/dL], aspartate transferase (AST) [U/L], alanine aminotransferase (ALT) [U/L], iron [µg/dL], ferritin [µg/L] and vitamin D [µg/L]. Last patient samples were collected in March 2021. This study was approved by the local ethics committee (study number B126201629776).

1.2 Serological assays

At diagnosis and at each follow-up visit, tTG IgA was routinely performed on serum samples (QUANTA Flash® h-tTG IgA, Inova Diagnostics, San Diego, USA on the BIO-FLASH® analyser, Inova Diagnostics) after which samples were frozen at -20°C. At the end of sample collection, tTG IgA, tTG IgG, DGP IgA and DGP were analysed in batch. Following assays were used for tTG analyses: QUANTA Flash® h-tTG IgA and QUANTA Flash® h-tTG IgG (Inova Diagnostics) and Celikey® IgA and Celikey® IgG (Thermo Fisher Scientific, Freiburg, Germany). Following assays were used for DGP analyses: QUANTA Flash® DGP IgA and QUANTA Flash® DGP IgG (Inova Diagnostics) and Gliadin^{DP} IgA and Gliadin^{DP} IgG (Thermo Fisher). QUANTA Flash® assays were performed on the BIO-FLASH® analyser (Inova Diagnostics), a fully automated CLIA analyser using a purified recombinant human antigen coated on paramagnetic beads. With the use of an isoluminol conjugate, a luminescent reaction occurs whose intensity is subsequently converted into chemiluminescent units (CU). All QUANTA Flash® results are classified positive from 20 CU (according to manufacturer weak positive between 20-30 CU). Celikey® and Gliadin^{DP}, both FEIA assays, were performed on the Phadia 200 analyser (Thermo Fisher Scientific), using enzyme-labed antibodies as conjugate. Fluorescence measurements are converted into EliA U/mL and all classified positive from 7 EliA U/mL (according to manufacturer dubious between 7-10 EliA U/mL).

1.3 Data analysis

Categorical data were reported as means and percentages and compared using chi-quare (χ^2 test); continuous data as medians and interquartile range (IQR) and compared used Mann-Whitney test. Normality of data was checked by D'Agostino-Pearson test: paired data with normal distribution were consequently compared with the paired samples t-test, as not normally distributed data, which cannot be transformed to a normal distribution, with the Wilcoxon test for paired samples. To estimate the mean time to serological remission, the Kaplan-Meier survival analysis was used and calculated as the area under the survival curve in the interval 0 to t_{max}; the Logrank test was used to compare the two survival curves. A p-value <0.05 was set as threshold for statistical significance. All statistical analyses were performed using MedCalc Statistical Software version 19.3 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2021).

2. Results

2.1 Patient demographics

After exclusion of patients with no informed consent (n=1) or clinical follow-up (n=1), a total of 13 patients were included, corresponding to 61 follow-up samples (three patients lacked the follow-up visit at t = 24 months, one other patient lacked follow-up visit at t = 6 months). An overview of demographic data completed with baseline histological, laboratory and clinical data is shown in table 1. Remarkable is the high percentages of the female patients (85%), the high prevalence of extra-intestinal symptoms and the low prevalence of biochemical/haematological abnormalities.

Table 1. Demographic, histological, clinical and laboratory data of included CD patients at time of diagnosis (t = 0)

DEMOGRAPHIC DATA	
Mean age, years (range)	40 (8-69)
Female, n (%)	11/13 (85%)
Adults, n (%)	11/13 (85%)
Risk factor	
Type I Diabetes mellitus, n (%)	1/13 (8%)
First-degree CD relatives, n (%)	1/13 (8%)
IgA deficiency, n (%)	0/13 (0%)
HISTOLOGICAL DATA	
Marsh 1, n (%)	1/13 (8%)
Marsh 2, n (%)	0/13 (0%)
Marsh 3A, n (%)	4/13 (31%)
Marsh 3B, n (%)	4/13 (31%)
Marsh 3C, n (%)	3/13 (23%)
Not applicable, n (%)	1/13 (8%)
CLINICAL DATA[+]	
Classic intestinal symptoms, n of patients score $\geq$ 1 (mean score of positive patients)	
Persistent diarrhoea*	9/12 (mean score 5.7)
Constipation*	5/12 (mean score 3.6)
Non-classic extra-intestinal symptoms, n of patients score $\geq$ 1 (mean score of positive patients)	
Anorexia/refusal to eat*	3/12 (mean score 8.3)
Loss-of-weight*	1/12 (mean score 9.0)
Vomiting*	2/12 (mean score 2.5)
Irritability*	7/12 (mean score 7.3)
Skin problems*	4/12 (mean score 4.5)
Aphtous stomatitis*	3/12 (mean score 5.6)
Headache**	6/11 (mean score 4.0)
Tiredness**	11/11 (mean score 7.0)
LABORATORY DATA[++]	
Anaemia, n (%)	1/12 (8%)
Elevated AST, n (%)	1/13 (8%)
Elevated ALT, n (%)	3/13 (23%)
Decreased iron, n (%)	0/13 (0%)
Decreased ferritin, n (%)	2/13 (15%)
Deficiency 25-OH-vitamin D, n (%)	3/13 (23%)
tTG IgA	
0 – 20 CU (<ULN)	0/13 (0%)
20 – 200 CU (<10xULN)	2/13 (15%)
>200 CU (>10xULN)	11/13 (85%)

[+]No rate of complaints on t = 0 available in *1/13 patients and **2/13 patients; [++]anaemia defined as haemoglobin <11 g/dL (<15 years old), <13 g/dL (men >15 years old), <12 g/dL (women >15 years old); elevated AST or ALT as >50 U/L (men), >35 U/L (women); decreased iron as <40 µg/dL (<18 years old), <33 µg/dL (>18 years old); decreased ferritin as <30 µg/L (men), <15 µg/L (women); deficiency 25-OH-vitamine D as ≤20 µg/L; ULN, upper limit of normal

2.2 Serological kinetic profiles

During the follow-up period, 7/13 patients were assessed to be 100% compliant to the GFD, 2/13 partially complaint (both only at t = 24 months non-compliant) and 2/13 non-compliant; data of GFD adherence was lacking for 2/13 patients (both children). The trends of kinetic profiles revealed for the corresponding serological assays of the two manufacturers were similar (attachment 1 and attachment 2). In all 100% compliant and partially compliant patients (n = 9/11), titers of tTG IgA and tTG IgG assays declined at every follow-up time point (less pronounced with the tTG IgG assays). Such decline was not found in the non-compliant patients (n=2/11). In 2/9 (partially) compliant patients and in 2/2 non-compliant patients, DGP IgA and DGP IgG antibodies were not consistently declining.

2.3 Correlation of serological assays with duodenal biopsy result

Of the 12 patients with a duodenal biopsy at time of diagnosis, 8 patients underwent a control histological assessment after 12 or 24 months with 6/8 being in complete histological remission (Marsh 0) (1/8 improved from Marsh 3c to Marsh 3a; 1/8 deteriorated from Marsh 3a to Marsh 3b) . When comparing serological results of these 6 patients between t=0 (Marsh 3a/b/c) and t=12 or t=24 (all Marsh 0), a significant decrease was noticed for all assays (*p=0.0313*, Wilcoxon test). The strongest decline was obtained for the tTG IgA assays (median difference Thermo Fisher assay 153.7 EliA U/mL (80-fold decrease); INOVA assay 1735.9 CU (76-fold decrease)), followed by the DGP IgG assay (median difference Thermo Fisher assay 73.4 EliA U/mL (24-fold decrease); INOVA assay 178.7 CU (28-fold decrease)), DGP IgA assay (median difference Thermo Fisher assay 58.0 EliA U/mL (4-fold decrease); INOVA assay 112.6 CU (19-fold decrease)) and tTG IgG assay (median difference Thermo Fisher assay 5.4 EliA U/mL (6-fold decrease); INOVA assay 27.9 CU (7-fold decrease)) (attachment 3).

2.4 Correlation with patient complaints

The number of intestinal and extra-intestinal complaints at diagnosis was highly patient dependent. In addition, 5/7 patients who fully adhered to the GFD described at one or more time points an increase in (extra-)intestinal symptoms. No correlation was found between (extra- and/or intestinal) symptoms and the various serological assays, neither in all patients (n = 13), nor in the 100% compliant patients (n = 7) (rank correlation, Spearman's rho <0,5).

2.5 Correlation with biochemical/haematological parameters

In 7/13 patients, limited abnormal biochemical/haematological parameters were found at diagnosis. Except for 25-OH-vitamin D deficiency, each abnormality normalized after t=3 months following GFD (except for 1/13 patients, normalization of ALT only after t=6 months).

2.6 Clinical interpretation of antibody titers

As can be observed in the total kinetic profiles of all patients together (attachment 2), the time point at which the serological result declines below the upper limit of normal (ULN) depended on both the assay used and the manufacturer's cut-off. An overview of the clinical interpretation of the various serological assays is shown in table 2. At diagnosis (t=0), the tTG IgG assay was significantly less sensitive for both Thermo Fisher (*p<0.001*) and INOVA (*p=0.002*) compared to the corresponding tTG IgA assay. At t = 6 months, significantly more patients had tTG IgA values below the ULN with the Thermo Fisher assay compared to the INOVA assay (10/12 vs. 3/12; *p=0.005*). The same was observed at the other time points, although not statistically significant (t=0, *p=0.317*; t=3, *p=0.071*; t=12, *p=0.052*; t=24, *p=0.057*).

Next, we harmonized the cut-offs for both assays at a predefined specificity of 100%,[19] by multiplying the cut-off of the Thermo Fisher assay with factor 2 (*i.e.* 14 EliA U/mL) and the INOVA assay with factor 5 (*i.e.* 100 CU). Using these cutoffs, the clinical interpretation for both manufacturers entirely synchronized from t=3 months.

Regarding DGP a significant higher amount of patients obtained a concentration below the ULN with the DGP IgA assay compared to the DGP IgG assay of INOVA at t=3 months (8/13 vs. 1/13, *p=0.005*), but not with the Thermo Fisher assay (6/13 vs. 2/13, *p=0.095*).

When comparing the tTG IgA vs. DGP IgA assay, the DGP IgA assay tended to normalize sooner compared to the tTG IgA assay with the INOVA assay (significant at t=3 and t=6 months; *p<0.001* resp. *p=0.045*). However, when using the Thermo Fisher assay, more patients were in serological remission from t=6 months with the tTG IgA assay compared to the DGP IgA assay, but not statistically significant (except t=24 months, *p=0.022*).

A final clinical interpretation of interest is the mean time to serological normalization in patients with GFD adherence (100% compliant and partially compliant patients, n = 9/13). Due to the low diagnostic sensitivity, this was not relevant for tTG IgG assays. Regarding tTG IgA, the results of Thermo Fisher normalized significantly sooner compared to the INOVA assay (Kaplan-Meier survival analysis, mean survival time 13.0 months [95% C.I. 10.0 to 16.0] vs. 19.8 months [95% C.I. 16.7 to 22.8] , *p=0.001*). However, after harmonization of cut-offs at a predefined specificity of 100%, the mean time to normalisation fully corresponded for both manufacturers (both 11.9 months [95% C.I. 9.1 to 14.8], *p=1.000*) (Figure 1). The DPG assays normalized later for the Thermo Fisher assay (16.2 months) but not for the INOVA assay (13.4 months), ditto for the DGP IgG assays (17.4 months for the Thermo Fisher assay, 17.3 months for the INOVA assay (attachment 4).

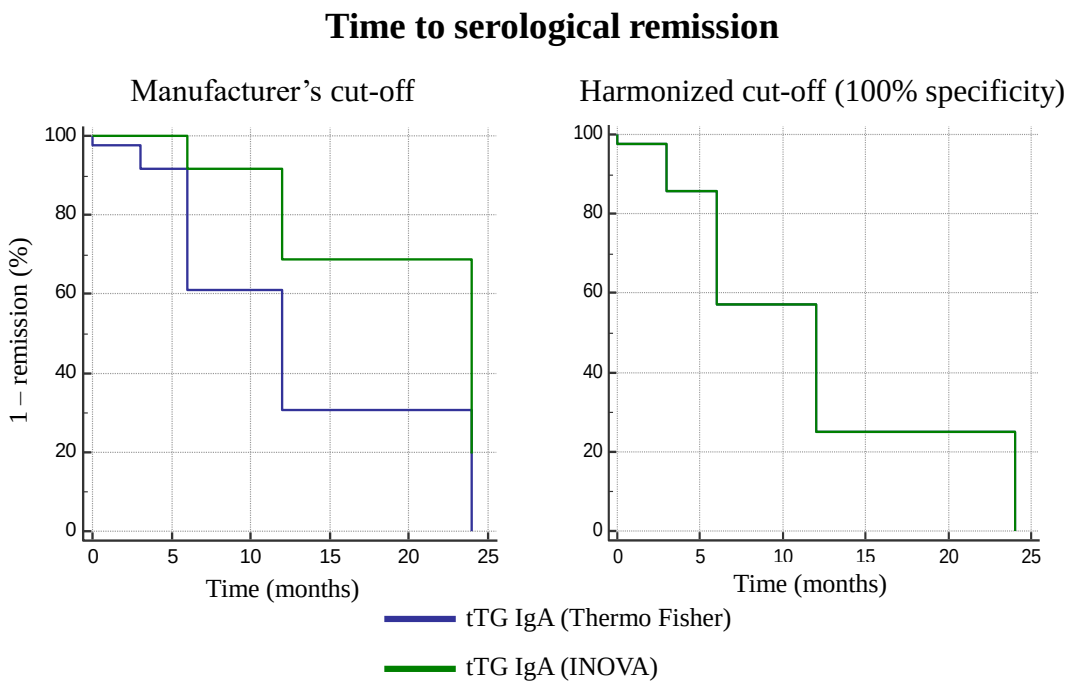

Figure 1. Time to serological remission of tTG IgA*. Left: time to normalization of the tTG IgA assay of Thermo Fisher and INOVA in 100% compliant and partially compliant patients (n = 9/13) based on the manufacturer's cut-off (Thermo Fisher 7 EliA U/mL; INOVA 20 CU). Right: time to normalization when using the harmonized cut-off (pre-defined 100% specificity) (Thermo Fisher 14 EliA U/mL; INOVA 100 CU)*

Table 2. Differences in clinical interpretation depending on used serological assay in all patients (n = 13). IN = INOVA; N° = number; TF = Thermo Fisher; ULN = upper limit of normal

	t = 0 months			t = 3 months			t = 6 months			t = 12 months			t = 24 months		
	N°	**<ULN**	**<2* (TF) or <5*(IN) ULN**	**N°**	**<ULN**	**<2* (TF) or <5*(IN) ULN**	**N°**	**<ULN**	**<2* (TF) or <5*(IN) ULN**	**N°**	**<ULN**	**<2* (TF) or <5*(IN) ULN**	**N°**	**<ULN**	**<2* (TF) or <5*(IN) ULN**
tTG IgA TF	13	1 (7.7%)	1 (7.7%)	13	3 (23.1%)	7 (53.8%)	12	10 (83.3%)	11 (91.6)	13	10 (76.9%)	12 (92.3%)	10	9 (90.0%)	9 (90.0%)
tTG IgG TF	13	11 [(1)] (84.6%)		13	12 (92.3%)		12	11 (91.6%)		13	13 (100.0%)		10	10 (100.0%)	
DGP IgA TF	13	1 [(2)] (7.7%)		13	6 [(2)] (46.2%)		12	6 [(2)] (50.0%)		13	6 [(2)] (46.2%)		10	4 [(2)] (40.0%)	
DGP IgG TF	13	0 (0.0%)		13	2 [(4)] (15.4%)		12	7 [(4)] (58.3%)		13	8 [(4)] (61.5%)		10	7 [(4)] (70.0%)	
tTG IgA IN	13	0 [(3)] (0.0%)	2 (15.4%)	13	0 [(3)] (0.0%)	7 (53.8%)	12	3 [(3)] (25.0%)	11 (91.6%)	13	5 [(3)] (38.5%)	12 (92.3%)	10	5 [(3)] (50.0%)	9 (90.0%)
tTG IgG IN	13	7 [(1)] (53.8%)		13	10 (76.9%)		12	11 (91.7%)		13	13 (100.0%)		10	10 (100.0%)	
DGP IgA IN	13	1 [(2)] (7.7%)		13	8 [(2)] (61.5%)		12	8 [(2)] (66.7%)		13	8 [(2)] (61.5%)		10	7 [(2)] (70.0%)	
DGP IgG IN	13	0 (0.0%)		13	1 [(4)] (7.7%)		12	7[(4)] (58.3%)		13	9 [(4)] (69.2%)		10	8 [(4)] (80.0%)	

* based on BOGAERT et al.[19]

[(1)] tTG IgG vs. tTG IgA for TF: at t=0 (***p<0.001****)*; tTG IgA vs. tTG IgG for IN: at t=0 (***p=0.002****)*

[(2)] DGP IgA vs. tTF IgA for TF: at t=0 (*p=1.000*), t=3 (*p=0.225*), t=6 (*p=0.327*), t=12 (*p=0.114*), t=24 (***p=0.223***); for DGP IgA vs. tTG IgA for IN at t=0 (*p=0.317*), t=3 (***p<0.001***), t=6 (***p=0.045***), t=12 (*p=0.249*), t=24 (*p=0.374*)

[(3)] tTG IgA TF vs. tTG IgA IN using manufacturer's cut-off: at t=0 (*p=0.317*), t=3 *(p=0.071)*, t=6 (***p=0.005***); t=12 (*p=0.052*), t=24 (*p=0.057)*

[(4)] DGP IgG vs. DGP IgA for TF: at t=3 (*p=0.095*), t=6 (*p=0.690*); t=12 (*p=0.443*), t=24 (*p=0.118*); for IN= at t=3 (***p=0.005***); t=6 (*p=0.690*); t=12 (*p=0.686*); t=24 *(p=0.615*)

All statistical differences calculated using χ^2 test

3. Discussion

Serological testing (particularly tTG IgA) is a crucial component in the diagnosis of CD. However, its role in the follow-up and clinical management of CD is much more restricted and less extensively documented. In this study, 13 newly diagnosed CD patients (11 adults, 2 children) were prospectively followed to investigate the kinetics of 8 serological assays: tTG IgA/IgG and DGP IgA/DGP manufactured by Thermo Fisher (FEIA) and INOVA (CLIA). The clinical interpretation of various assays in addition to the correlation between the serological results and the patient's symptoms, adherence to the GFD and biochemical/haematological abnormalities were investigated.

Despite the low amount of included patients, a significant difference in clinical interpretation of the tTG IgA assay of Thermo Fisher compared to the tTG IgA assay of INOVA was noticed when using the manufacturer's cut-off (7 EliA U/mL resp. 20 CU). A recent publication by BOGAERT and colleagues[19] showed that the tTG IgA ULN corresponded to a sensitivity and specificity of 89% and 99% for Thermo Fisher and to 90% and 98% for INOVA, respectively, with a similar area under the curve (AUC) to distinguish CD patients from controls. By aligning the cut-offs of both assays to a specificity of 100%, *i.e.* multiplying the ULN for Thermo Fisher with 2 (14 EliA U/mL) and with 5 for INOVA (100 EliA U/mL), both assays achieved an sensitivity of 82%. This confirms recent literature showing that selecting thresholds based on a pre-defined specificity (or likelihood ratio (LR) or post-test probability) is a better alternative to harmonize interpretation between different assays.[17–19,24] Applying the harmonized cut-offs (corresponding to 100% specificity) aligned the clinical interpretation in terms of serological remission after initiating the GFD, revealing an equal mean time to serological remission at 11.9 months (95% C.I. 9.1 to 14.8).

This time to normalization is in line with other studies. Several studies investigated whether or not CLIA assays normalize sooner compared to ELISA assays and revealed conflicting results. SANSOTTA et al.[30] found in a retrospective study in children with CD (no information on GFD adherence) a median time of 14.7 months for tTG IgA normalization using the CLIA assay of INOVA (n=150 children, median follow-up time 31.9 months); using the ELISA assay of Thermo Fisher, a faster median time of 11.7 months for tTG IgA normalization was obtained (n=150 other children, median follow-up time 23.3 months). They presumed that CLIA was a more sensitive and accurate method compared to ELISA in detecting gluten contamination and that both assays could not be used interchangeably, neither at diagnosis nor during follow-up. However, it should be noted that no harmonized cut-offs are used between the manufacturer's assays. A similar study[36] in 42 children on GFD (no data on GFD adherence) found in contrary a significant higher percentage decrease in antibody level using CLIA assays (tTG IgA and DGP IgA + IgG both of INOVA) compared to ELISA (tTG IgA INOVA) at 4 and 12 months after start of the GFD. However, more patients were serologically normalized with the DGP IgA + IgG assay compared to the other assays, indicating that after gluten withdrawal, antibodies that recognize modified DGP gluten antigens declined first, whereas autoantibodies against tTG persisted longer.[21,36] This faster normalization of DGP IgA compared to tTG IgA was also confirmed in our study for the INOVA assays, but not for the Thermo Fisher assay when applying the manufacturer's cut-off (table 2).

An important question is if a serological result can be used as surrogate marker for adequate GFD adherence. In our study, too few patients were partially- (n=2) or non-compliant (n=2) to the GFD, so no firm conclusion could be made. Another limitation of our study was the lack of GFD adherence assessment by a professional nutritionist. However, this question was comprehensively studied in the paper by LEFFLER et al.,[21] who calculated in 107 adults on a GFD ≥ 1 year a PPV for non-compliance to GFD of tTG IgA and DGP IgA (both ELISA assays of INOVA) of only 53.8% resp. 50.0% ('fair to poor GFD' adherence, assessed by a professional nutritionist). The NPV was higher for both assays (84.5% resp. 86.7%). Areas under the receiver operating characteristic (AU ROC) curves were unsatisfactory for both assays (0.721 resp. 0.789). Additionally, they noticed that this PPV was even lower when including patients <1 year on a GFD too (n = 150) (30.6% for tTG IgA and 30.6% for DGP IgA). The same conclusion was reached in the study of SUGAI et al.,[32] in which 82 newly diagnosed adult CD patients were followed for 12 months. Both tTG IgA and DGP IgA assays (ELISA assays of INOVA) had a low PPV (53.1% resp.

47.7), but slightly higher NPV (74.2% resp. 62.2%) in detecting partially adherence to a GFD, with a low corresponding AU ROC to discriminate strict from partially compliant patients (0.72 resp. 0.66). Follow-up studies in children found similar low sensitivities for tTG IgA in monitoring compliance with the GFD, results for DGP IgA were slightly higher (24% resp. 60%).[37] Of interest, we observed that in 2/7 of our 100% compliant patients (patient 2 and patient 3, attachment 1), a clear increase in DGP IgA/IgG levels was noticed between t = 6 months and t = 12 months despite a decrease in tTG levels. Unfortunately, no follow-up biopsy was available in these patients.

This indicates that in the first year after starting a GFD, the general trend is more informative to a clinician compared to the actual antibody titer (*i.e.* are antibody levels declining?). After this "transition period" and normalization of the antibody titer, a subsequent increase in tTG IgA (or DGP IgA) is a good indicator of gluten ingestion.[38] Serology in strictly compliant patients tended to decrease even further for multiple years.[31] However, a clinician must be aware that a substantially amount of non-compliant patients have a falsely negative serological result[21] and serology cannot detect minor traces or intermittent consumption of gluten, making normal antibody titers not sensitive to detect ongoing gluten exposure.[38] Additional important information here is the knowledge regarding the biological variation of these antibodies. One study calculated in 28 CD patients on a GFD the reference change value (RCV) of tTG IgA to be 55.5% (using Thermo Fisher's FEIA assay on the Immunocap 250 analyzer with analytical imprecision of 5.7%; within-subject coefficients of variation (CV_I) 19.2%; between-subject biological variations (CV_G) 75.6%).[39] This indicates that if the titer of tTG IgA in a CD patient following a GFD increases more than 55.5%, then it is 95% certain that this rise is not caused by purely biological variation, but that there is a high probability of gluten exposure. The use of a RCV can be used to detect significant differences between serial quantitative measurements.

Regarding the ability of serological assays to detect persistent villous atrophy in patients on a GFD, a recent meta-analysis based on 26 publications estimated the sensitivity of tTG IgA at 50% (specificity 83%; no difference depending on assay type, biopsy method or patient age) with an AUC ROC in adults of 0.781 and in children of 0.879. These results are in marked conflict compared to the high sensitivity and specificity for the diagnosis of CD.[9,13,14] Results of DGP IgA assays (based on 2 publications) are equal with low sensitivity but higher specificity.[34] For a clinician, the key message here is essentially the same as above: a continually positive serological results despite one year of GFD is highly specific for persistent villous atrophy (and non-compliance to the GFD), but a negative serological results does not implicate a normalized duodenal architecture (or fully adherence to the GFD). Still, there is no consensus if patients who fully adhere to a GFD and who clinically respond to the GFD and have normalized serological titers need a repeated duodenal biopsy to investigate the complete healing of the intestinal mucosa.[38] Counterarguments for these 'healthy' patients are that it can take almost a decade for mucosal recovery to occur[40] and that no differences in long-term symptoms (10 year) or well-being is found in patients with or without follow-up biopsies.[41]

Since serological assays lack sensitivity to detect non-adherence to the GFD or persistent mucosal damage, new biomarkers are approached to detect (un)intentional gluten exposure. One of these are monoclonal antibodies detecting gluten immunogenic peptides (GIP). These peptides are resistant to gastro-intestinal digestion and cause an immunogenic reaction in T-lymphocytes of celiac patients. By detecting them in urine or faeces, a direct and quantifiable assessment of gluten exposure can be made.[42,43] Results are promising and are both useful in diagnosis (to confirm gluten exposure making serological and histological result reliable) and follow-up, in addition to a clinical assessment, serological results and assessment by a professional dietician. However, sensitivity to detect mucosal damage in follow-up, similar to serological assays, is low with comparable specificity too.[44]

To conclude, follow-up of patients with celiac disease, even if they are asymptomatic, is crucial to avoid long-term complications.[45] Patients must be encouraged to visit their gastro-enterologist, general practitioner and professional nutritionist on a regular basis. Clinicians can use serological assays (tTG IgA or DGP IgA/IgG) in the

first year after diagnosis to assess the general trend of decreasing antibody levels and thereafter to detect an increasing serological titer. It is important that the serology is used in addition to the clinical assessment and that the limited sensitivity to detect fully adherence to the GFD and histological damage is taking into account. Laboratories need to inform the clinician about the diagnostic performance characteristics of the provided assay and can provide additional recommendations based on harmonized cut-offs with a pre-defined specificity, by using LR or by post-test probabilities.[19] Although the number of included patients in this prospective study is low, the study shows that the clinical interpretation between assays from different manufacturers can be improved significantly after harmonizing the used cut-offs based on a pre-defined specificity.

TO DO/ACTIONS

1) Clinicians must be aware of the inter-manufacturer differences and should rely on cut-offs with a pre-defined specificity to harmonize clinical decision making.

2) Future prospective studies including a higher number of patients, are warranted to investigate the performance characteristics of both FEIA and CLIA assay in the follow-up of coeliac patients by using harmonized cut-offs.

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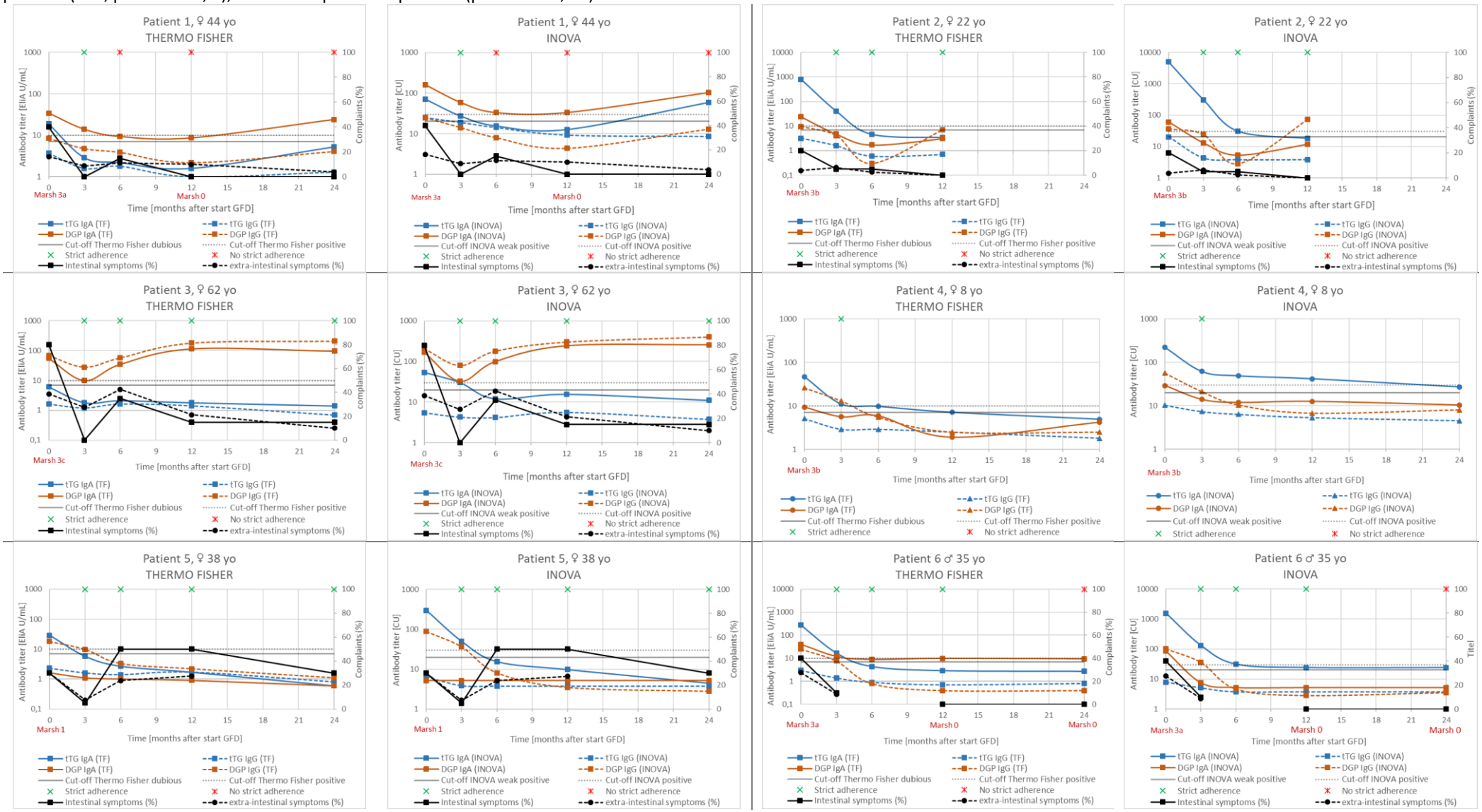
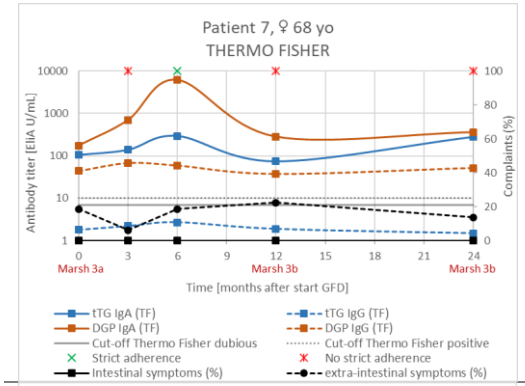
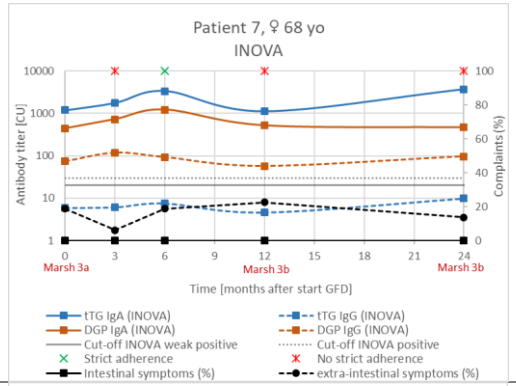
ATTACHMENTS

Attachment 1: Serological kinetics of all patients (n=13): 100% compliant patients (n=7, patient n° 2, 3, 5, 8, 10, 11, 12); partially compliant patients (n=2, patient n° 6, 9); non-compliant patients (n=2, patient n° 1, 7); data incomplete in 2 patients (patient n° 4, 13)




Patient 7, ♀ 68 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Marsh 3a
Marsh 3b
Marsh 3b
Time [months after start GFD]
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)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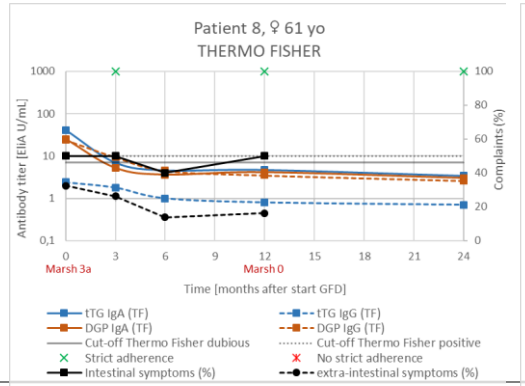
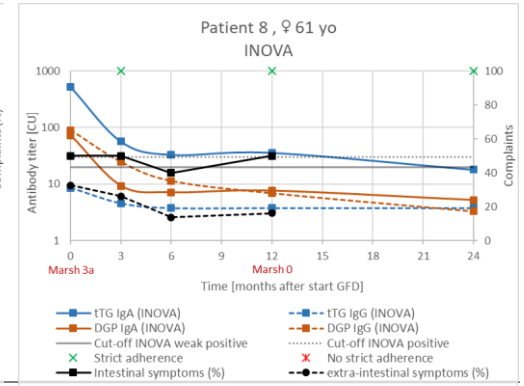
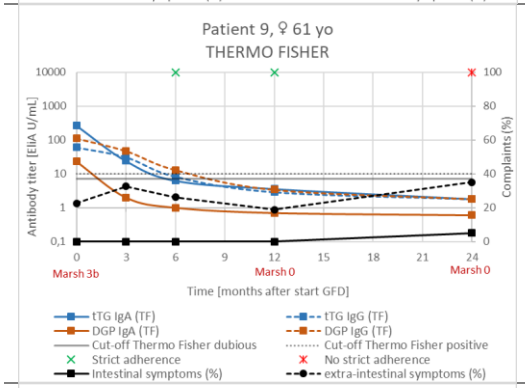
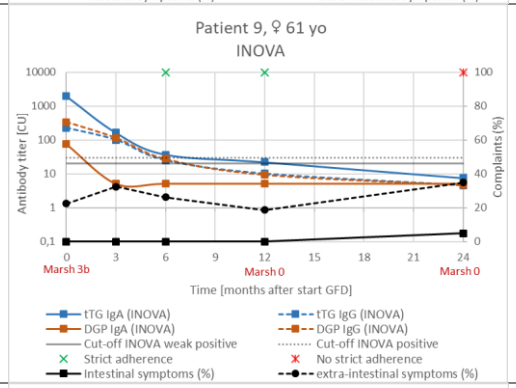
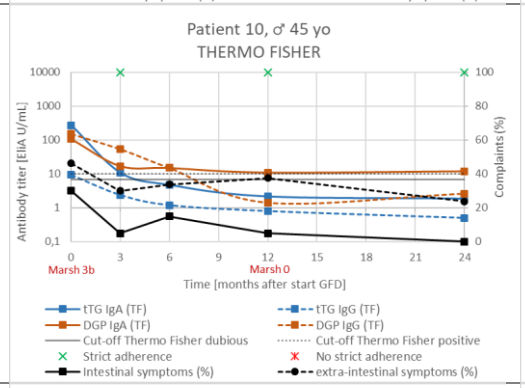
INOVA
Antibody titer [CU]
Complaints (%)
Marsh 3a
Marsh 3b
Marsh 3b
Time [months after start GFD]
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

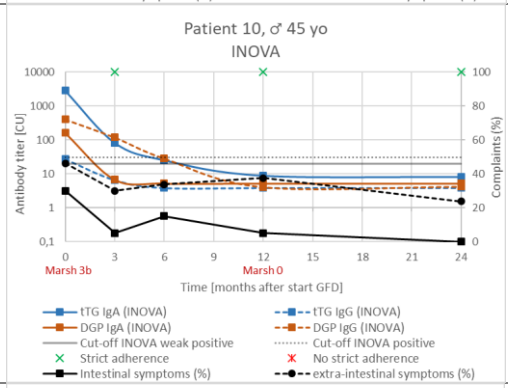

Patient 8, ♀ 61 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Marsh 3a
Marsh 0
Time [months after start GFD]
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

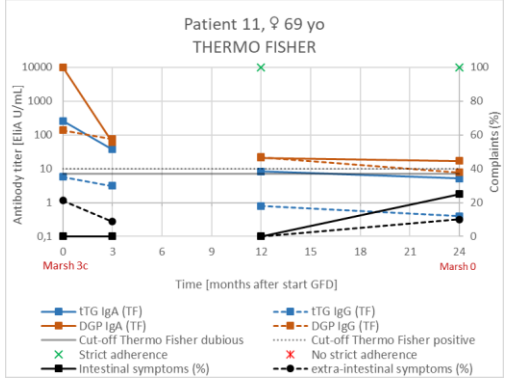

Patient 8 , ♀ 61 yo
INOVA
Antibody titer [CU]
Complaints
Marsh 3a
Marsh 0
Time [months after start GFD]
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

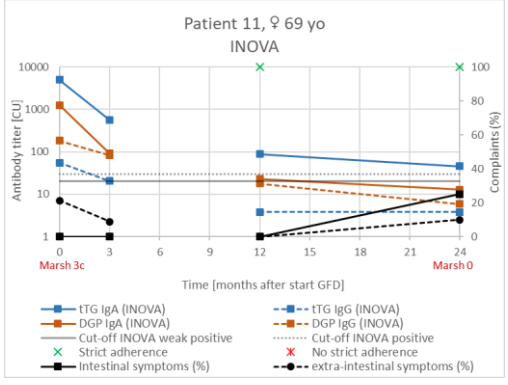

Patient 9, ♀ 61 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Marsh 3b
Marsh 0
Marsh 0
Time [months after start GFD]
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

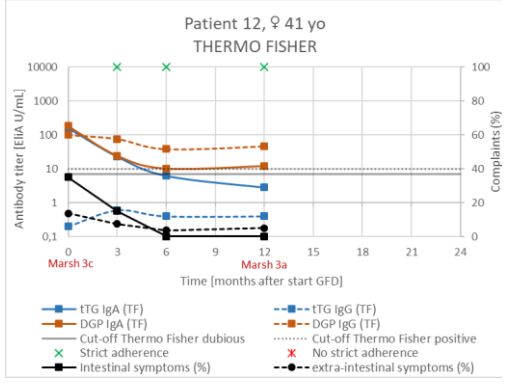

Patient 9, ♀ 61 yo
INOVA
Antibody titer [CU]
Complaints (%)
Marsh 3b
Marsh 0
Marsh 0
Time [months after start GFD]
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

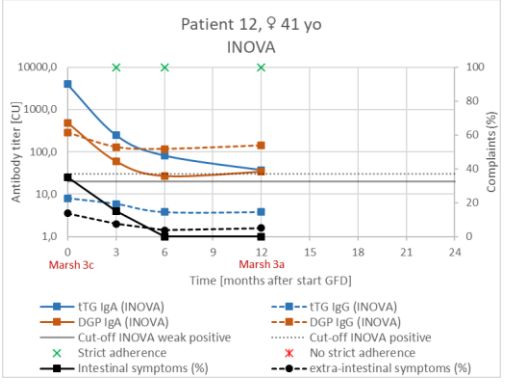

Patient 10, ♂ 45 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Marsh 3b
Marsh 0
Time [months after start GFD]
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

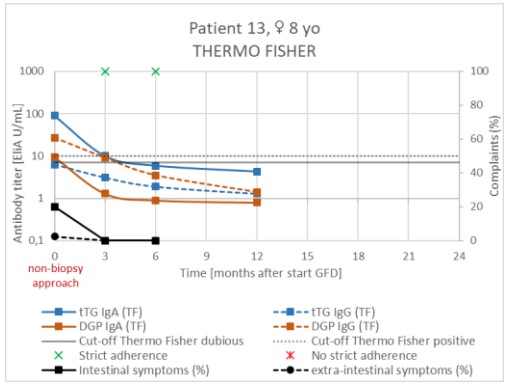

Patient 10, ♂ 45 yo
INOVA
Antibody titer [CU]
Complaints (%)
Marsh 3b
Marsh 0
Time [months after start GFD]
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

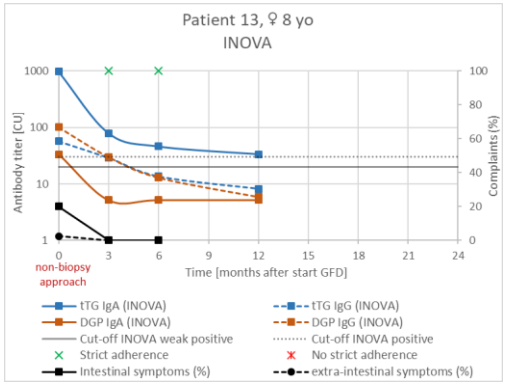

Patient 11, ♀ 69 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Marsh 3c
Marsh 0
Time [months after start GFD]
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)


Patient 11, ♀ 69 yo
INOVA
Antibody titer [CU]
Complaints (%)
Marsh 3c
Marsh 0
Time [months after start GFD]
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

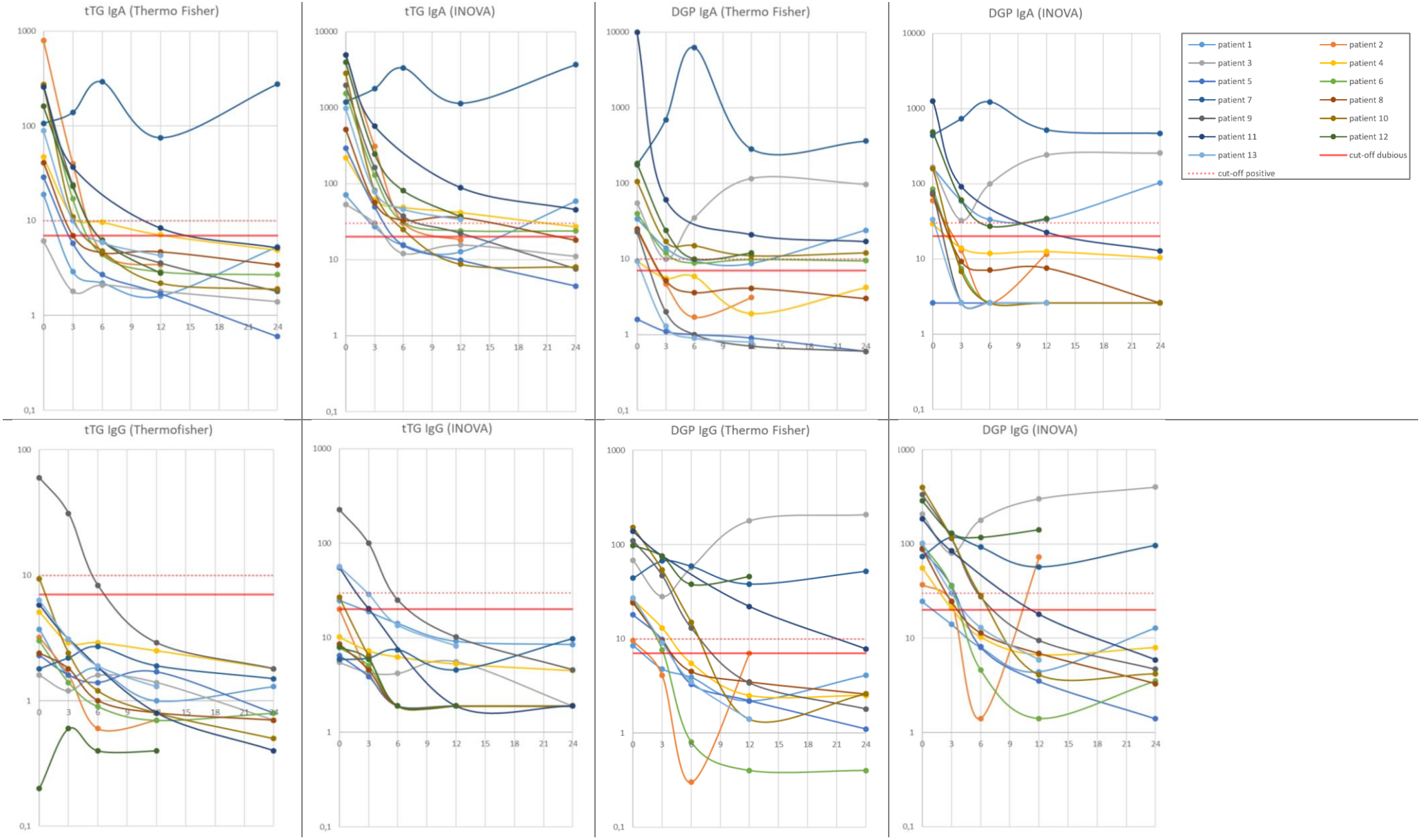

Patient 12, ♀ 41 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Marsh 3c
Marsh 3a
Time [months after start GFD]
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)


Patient 12, ♀ 41 yo
INOVA
Antibody titer [CU]
Complaints (%)
Marsh 3c
Marsh 3a
Time [months after start GFD]
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)


Patient 13, ♀ 8 yo
THERMO FISHER
Antibody titer [EliA U/mL]
Complaints (%)
Time [months after start GFD]
non-biopsy approach
tTG IgA (TF)
tTG IgG (TF)
DGP IgA (TF)
DGP IgG (TF)
Cut-off Thermo Fisher dubious
Cut-off Thermo Fisher positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)


Patient 13, ♀ 8 yo
INOVA
Antibody titer [CU]
Complaints (%)
Time [months after start GFD]
non-biopsy approach
tTG IgA (INOVA)
tTG IgG (INOVA)
DGP IgA (INOVA)
DGP IgG (INOVA)
Cut-off INOVA weak positive
Cut-off INOVA positive
Strict adherence
No strict adherence
Intestinal symptoms (%)
extra-intestinal symptoms (%)

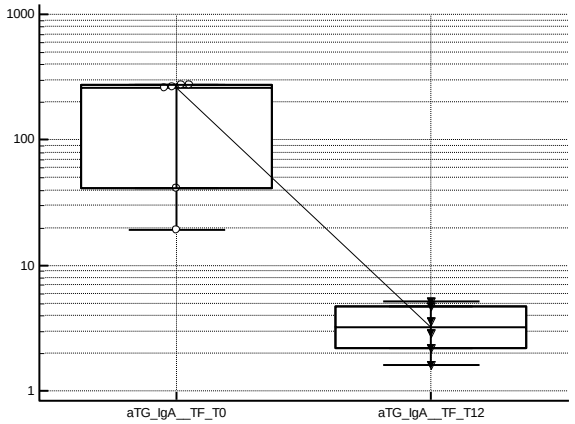
Attachment 2: total kinetics of all patients (n=13)

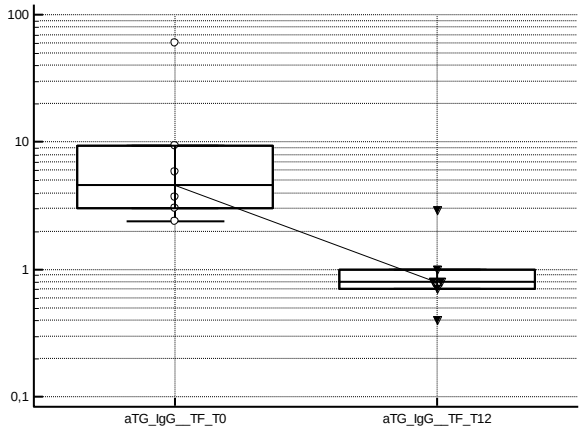


Attachment 3: Different box-and-whiskers plots of patients with Marsh 3a/b/c at T0 and repeat biopsy at T12 or T24 with Marsh 0, n = 6

1. Thermo Fisher assays

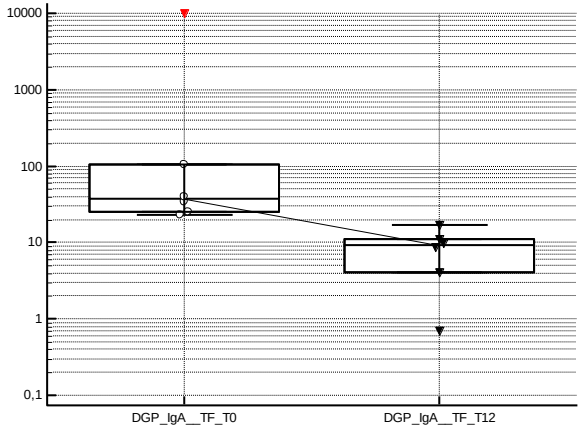
tTG IgA
Median difference
-153.7 EliA U/mL
[95% C.I. -271.1 to -17.4]

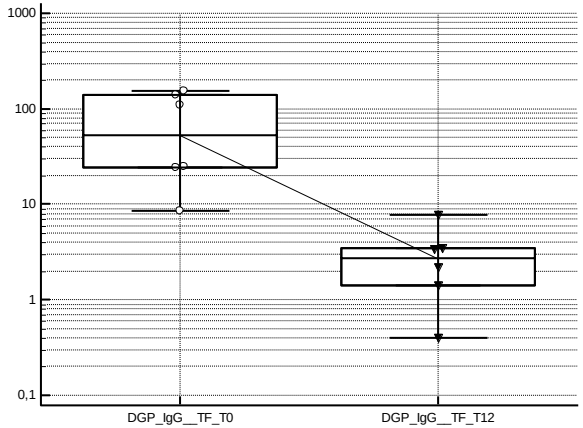




tTG IgG
Median difference
-5.4 EliA U/mL
[95% C.I. -57.1 to -1.6]

DGP IgA
Median difference
-58.0 EliA U/mL
[95% C.I. -9982.0 to -20.9]

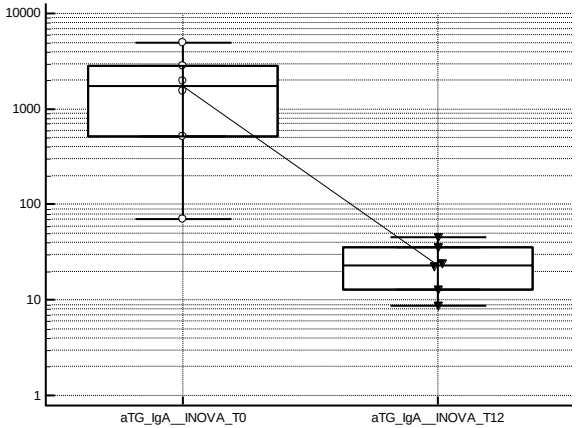


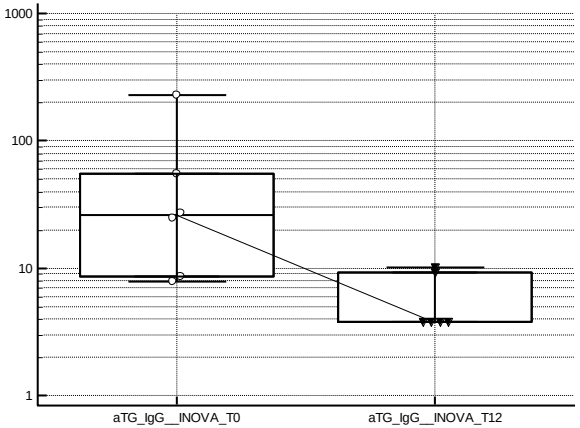


DGP IgG
Median difference
-73.4 EliA U/mL
[95% C.I. -151.6 to -6.3]

2. INOVA assays

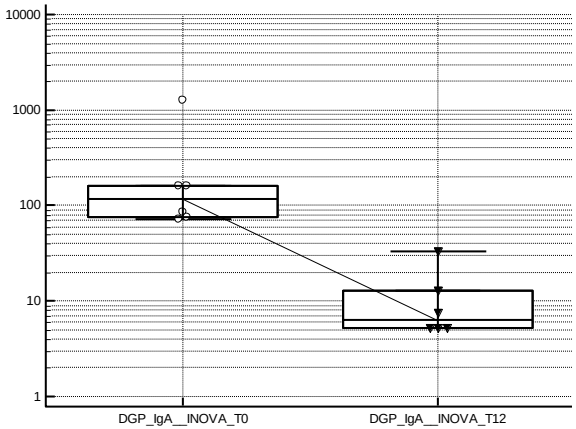
tTG IgA
Median difference
-1735.9 CU
[95% C.I. -4920.1 to -58.2]

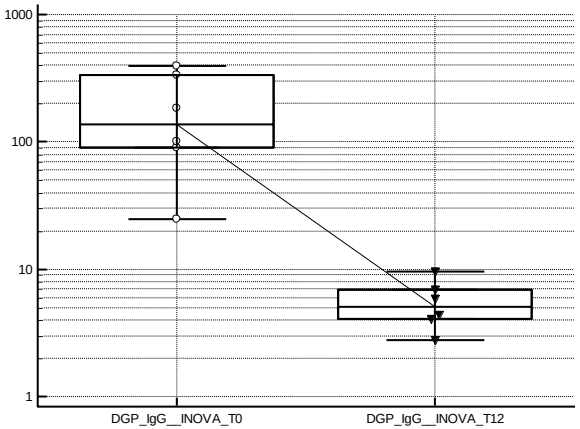




tTG IgG
Median difference
-27.9 CU
[95% C.I. –217.4 to -4.1]

DGP IgA
Median difference
-112.6 CU
[95% C.I. –1251.5 to -64.6]

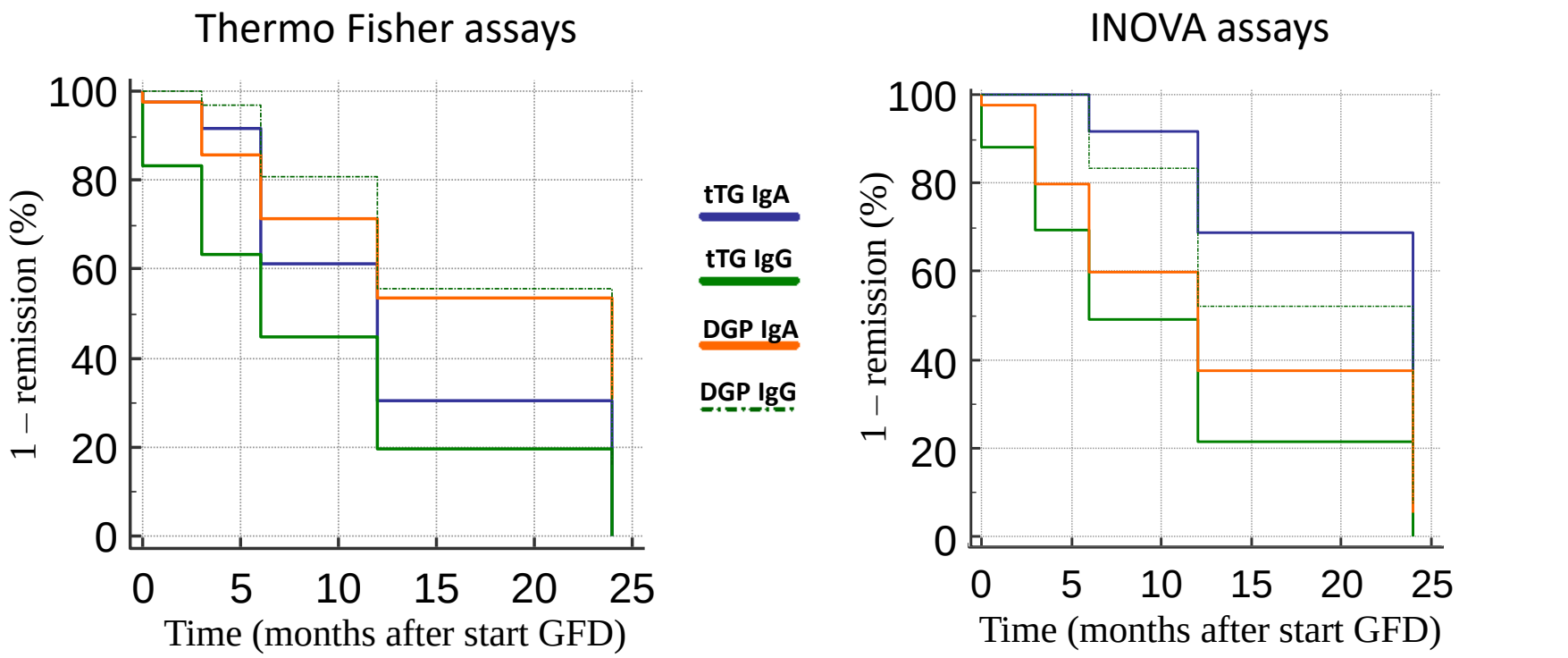




DGP IgG
Median difference
-178.7 CU
[95% C.I. -393.1 to -20.2]

Attachment 4: Kaplan-Meier survival analysis of 100% compliant and partially compliant patients (n = 9)

Time to serological remission



Mean time to serological remission
tTG IgA: 13.0 months (95% C.I. 10.0 to 16.0)
tTG IgG: 9.4 months (95% C.I. 6.8 to 12.1)
DGP IgA: 16.2 months (95% C.I. 12.9 to 19.6)
DGP IgG: 17.4 (95% C.I. 14.2 to 20.7)

Mean time to serological remission
tTG IgA: 19.8 months (95% C.I. 16.7 to 22.8)
tTG IgG: 10.3 months (95% C.I. 7.5 to 13.0)
DGP IgA: 13.4 months (95% C.I. 10.2 to 16.6)
DGP IgG: 17.3 months (95% C.I. 14.0 to 20.5)