Purified oat protein can trigger acute symptoms linked to immune activation in coeliac disease patients: implications for oats safety in the gluten-free diet

Short title: Immune and symptomatic responses to oat avenin

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Keywords: Oats, avenin, coeliac disease, T cells, Interleukin-2

Abstract (250 words)

Objective Oats ingestion in coeliac disease (CD) is generally regarded as safe although oats can trigger T cells specific for oat avenin in the gut and blood of some. To correlate immune and clinical outcomes to oats, short and longer-term feeding studies were performed using purified avenin and oats to examine symptoms, T-cell immunity, and intestinal histology in CD.

Design 33 treated HLA-DQ2.5+ adult CD patients underwent single-bolus, 6-week oat avenin, or 3-month whole oats ingestion. Avenin-specific T cell activation was measured using serum interleukin-2 (IL-2), a sensitive and specific biomarker of gluten-induced T cell

activation and symptoms in CD. Symptoms, intestinal histology and immune studies on blood and duodenum were undertaken.

Results Avenin induced dose-dependent T cell activation in 38% and acute symptoms in 59% of CD patients. Higher IL-2 levels correlated with more severe symptoms. A single highly symptomatic patient vomited to avenin (1/29; 3%) and exhibited a striking pro-inflammatory cytokine profile similar to wheat-induced responses. Avenin increased the frequency of CD38-expressing tetramer+integrin β7+ T effector memory CD4+ T cells in the blood but symptoms, IL-2 release and tetramer frequency fell following 6-week oat avenin intake and no enteropathy was observed.

Conclusions Purified gluten-free oats can trigger acute immune activation and symptoms in a dose-dependent manner but generally at a level insufficient to cause sustained symptoms or small bowel enteropathy. In 3% of cases, oat avenin triggered a pro-inflammatory wheat-like response, indicating that clinical follow-up is important and a minority of CD patients may need to exclude oats.

1. What is already known on this topic?

- Oat feeding studies suggest most individuals with coeliac disease (CD) can safely tolerate them although some patients harbour proinflammatory T cells in the gut and blood that are triggered by avenin, the gluten-like protein in oats
- Oats are a highly nutritious cereal associated with improved cardiovascular and metabolic indices so their addition to the highly restrictive gluten-free diet is appealing
- The discovery of serum interleukin-2 (IL-2) as a biomarker of gluten induced T cell activation and symptoms provides a sensitive and specific tool to assess oat safety

2. What this study adds

- Almost a third of CD patients experience acute T-cell activation linked to adverse symptoms when they consume oat avenin in a dose-dependent manner however this is rarely associated with intestinal mucosal damage or serologic deterioration following 6-week ingestion
- In 3% of cases, CD patients are super-sensitive to oats and display acute symptoms such as vomiting and a pro-inflammatory cytokine response resembling that induced

by wheat gluten, suggesting the need for follow-up and individualised dietary recommendations

3. How this study might affect research, practice, or policy

- The findings support the overall safety of oats for most patients with CD but indicate careful clinical follow-up is important
- Severe adverse symptoms to oats such as vomiting may be a marker of wheat-like pro-inflammatory responses and suggests avoidance of oats by that individual is prudent
- At a food regulatory level it remains important that food labelling clearly defines the presence of oats and enables CD patients to make an informed choice to exclude oats from their gluten-free diet

INTRODUCTION

Coeliac disease (CD) is a prevalent immune-mediated enteropathy precipitated by exposure to dietary gluten.[1] Central to its development are pro-inflammatory CD4+ T cells that recognise gluten peptide epitopes from prolamin proteins within wheat, barley and rye.[2] Dietary avoidance of these proteins is important for mucosal healing, symptom resolution, and reduced risk of complications.[3]

Oats was considered unsuitable for people with CD based on early feeding studies[4] but contemporary data employing oats free of gluten-contamination generally support their safety.[5] While most countries allow oats as part of the gluten-free diet (GFD), Australian and New Zealand food legislation excludes oats citing the lack of safety data. Uncertainty regarding oats safety in CD persists for several reasons. Firstly, *in vitro* and *in vivo* immune studies have revealed the oats-derived prolamin, avenin, contains peptides capable of stimulating pro-inflammatory avenin-specific T cells in the blood and duodenum of some people with CD.[6-8] We showed 6/73 (8%) of CD patients mounted an avenin-specific T cell response in blood six days after commencing 3-day oat ingestion and that the DQ2.5-ave-1a-c T cell epitopes (PYPEQEQPI/F) share close homology to the immunogenic barley-derived T cell epitope DQ2.5-hor-3a (PIPEQPQPY).[7] Secondly, feeding studies have shown contamination-free oats can induce villous atrophy or increase mucosal inflammation.[6, 9-11]

Thirdly, reports of adverse symptoms after oats in CD are reported by some patients but it remains unclear if they are due to the avenin or other factors such as the high fibre load.

Resolving oat safety in CD is important as there is a need to optimise the quality of the GFD and oats are a highly nutritious cereal. We sought to address this question by leveraging the strength of two novel developments. Firstly, a novel immune platform that we and colleagues developed based on interleukin-2 (IL-2) production in serum 4 hours after single-dose gluten ingestion that is now validated as a highly sensitive and specific readout of gluten induced T cell activation and symptoms.[12-18] Secondly, we developed an approach to extract food-grade avenin from contamination-free oats to enable high-dose feeding studies, free from the confounding effects of the high fibre and FODMAP load present in large doses of whole oats.[19] Armed with these two innovations, here we report a series of avenin and oat feeding studies to examine the relationship between oat-induced symptoms, immune activation and clinical outcomes.

MATERIALS AND METHODS

Study participants

CD patients aged between 18-70 years of age were recruited (Supplementary Table 1). Participants were enrolled from April 2019 to March 2022. A diagnosis of CD was based on the presence of small intestinal villous atrophy associated with positive CD serology and a supportive clinical history. To support the inclusion of patients with well-treated disease, participants needed to be adherent to a GFD for at least 12 months and have transglutaminase (tTG)-IgA and deamidated gliadin peptide (DGP)-IgG levels no greater than 10% above the upper limit of normal.

Patient and public involvement

Several of the authors have had a longstanding relationship with national patient association Coeliac Australia and have been involved in regular speaking events such as public Gluten Free Expos and patient forums. An identified area of need for CD patients is clarifying the safety of oats, especially in Australasia, where oats are excluded from the GFD. The design of the study was informed by CD patient feedback.

Baseline assessment

Baseline blood was collected for tTG-IgA and DGP-IgG (Melbourne or Dorevitch Pathology) and HLA typing was performed in house as described.[20]

Avenin protein

Oats (Cv. *Wandering*) were sourced from a dedicated gluten-contamination-free farm (Western Australia) that rigorously followed the oats purity protocol. Purified avenin was extracted in a gluten-contamination free food-grade facility as we previously reported.[19] 2.1kg of avenin was isolated from 400kg of contamination-free oats. The avenin protein was confirmed to contain the published avenin-specific T cell epitopes,[19] were free of contaminants including heavy metals and low in FODMAPs, with levels < 0.02g/6g serve (Monash University).

Avenin dose-escalation, 6-week and 3-month feeding studies

A dose-escalation study employed sequential single-bolus avenin ingestion each with an intervening 4-week washout period. Avenin protein was added by the trial nurse into 100mL liquid such as chocolate flavoured milk and mixed vigorously in shaker bottles prior to consumption. Avenin dose commenced at 0.1g avenin (equivalent to approximately 10g oats), followed by 0.5g, 1g, 4g, and 6g. Blood for serum isolation and IL-2 testing was collected immediately prior to each challenge and 4 hours after. For participants who were highly symptomatic and/or had a high IL-2 response at 0.1g avenin, the subsequent dose tested was 0.05g. For the 6-week feeding study, avenin was provided to participants in single dose containers and consumed daily as above. For the wheat control patient, 10g of vital wheat gluten was consumed daily for 6 weeks. For the 3-month oat feeding study, the same variety of whole oats used to isolate the avenin (Cv. *Wandering*) was provided to participants in vacuum packed sealed bags to maintain freshness, with 50g consumed as porridge daily.

Symptom diaries

Self-reported symptom severity was recorded using a modified version of the Celiac Disease Patient-Reported Outcome tool (CeD PRO®).[21] For the dose-ranging study, patients responded to the prompt, “Thinking about your worst experience in the past 1 hour, how severe was each of the following symptoms?” within an hour before, and at 1, 2, 3, and 4 hours after gluten challenge. A whole number rating on a scale from 0 (no symptoms) to 10 (worst possible severity) was recorded individually for abdominal cramping, bloating, gas,

pain, nausea, diarrhoea, loose stool, headache, and tiredness. During the 6-week challenge patients recorded symptoms scores at baseline and then daily, with a run-in period to identify baseline symptoms prior to commencement.

Blood collection and cell isolation

Blood was collected for serum at baseline and 4h after avenin challenge in the dose-escalation study and similarly at the beginning and end of the 6-week avenin feeding study for IL-2 and proteomics. Blood was allowed to clot for 1-2h after which it was centrifuged at 2000 x g for 10mins and serum stored at -80ºC. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood at 3 time points (baseline prior to 6-week challenge, Day 6, and at follow-up within 5 days of the 6 week endoscopy) using Ficoll-Paque™Plus density-gradient centrifugation (GE Healthcare) using Leucosep™ tubes (Greiner Labortechnik, Kresmuster, Austria).

Duodenal tissue collection

Duodenal tissue was collected by gastroscopies at baseline and the end of the challenge. A total of 12 spaced duodenal biopsies were collected from the 2nd part of the duodenum and 4 from the 1st part. Histomorphometry reported villous height: crypt depth ratio and IEL count on FFPE tissue (Envoi Pathology, Queensland). Lamina propria mononuclear cells (LPMC) were isolated from six duodenal biopsies using enzyme digestion. Briefly, biopsies epithelial cells were removed using 2mM EDTA in 3% FBS/PBS. Biopsies were incubated with RPMI1640 containing 3% FBS, 1mg/mL collagenase (type IV, Worthington or Type H Sigma) and 50µg/mL DNAse I (Roche) for 40-60mins at 37ºC with gentle mixing. Biopsies were vortexed and passed through a 70µM cell strainer with a syringe plunger and washing.

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Serum interleukin-2

IL-2 levels were measured using electrochemiluminescence with V-plex or S-plex kits following manufacturer's instructions (Meso Scale Discovery, MSD). Plates were run on a SQ120MM instrument. Mean cytokine levels from duplicate wells were analysed using MSD Discovery Workbench software. Based on our prior published work cytokine concentrations for values below the LLOD were reported as equal to the LLOD. Whist we have shown a 2-fold IL-2 elevation between baseline and 4 hours is sensitive and specific for gluten-specific T cell responses in CD after wheat ingestion,[12] we adopted a more conservative 2.5-fold cut-

off in this study as avenin challenges had never been undertaken and this would ensure that increases over 2.5-fold were more likely to be significant.

O-link proteomic screen

Protein targets were quantified in multiplex with the Proximity Extension Assay at the WEHI Genomics facility using the Olink Target platform and Inflammation panel (Olink Proteomics, Sweden). Additional information is in Supplementary Materials.

Tetramer and phenotypic staining and avenin IgA ELISA

Detailed information is in Supplementary Materials.

Statistical analysis

Summary statistics, median and IQR, were presented for continuous data, and as frequencies and percentage for categorical data. Comparisons between two matched groups were performed using Wilcoxon matched pairs signed rank tests. P values <0.05 were considered significant. Statistical analyses were performed using Prism software version 9 (Graphpad).

RESULTS

Study participants

Outline of the single-bolus and longer-term feeding studies with participant flow is illustrated in Figure 1. A total of 33 treated CD patients participated in this study (Table 1). They were aged between 22-70 years (median 49 years) and 29 (88%) were females. All participants were HLA-DQ2.5+, carrying both *HLA-DQA1*05* and *HLA-DQB1*02*.

Table 1. Characteristics of the study population.

Details	**Participants (n=33)**
Median age (IQR), y	49 (43-63)
Median age at diagnosis (IQR), y	40 (31-47)
Median time on GFD (IQR), y	9 (5-14)
Females, n (%)	29 (88)
HLA genotype, n (%)	
DQ2.5/X	26 (79)
DQ2.5/DQ2.5	7 (21)
Baseline assessments	
tTG-IgA serology (negative %)	97
DGP-IgG serology (negative %)	100

Oat avenin induces dose-dependent acute immune activation and symptoms

Twenty-nine treated CD patients completed the single-bolus feeding study (Figure 1). They had been following a GFD for a median of 112 months (range 12-396) and 28/29 (97%) had negative CD serology at study entry. The cohort included four participants (#CD27, #CD28, #CD29, and #CD14) who had positive T cell responses following 3-day oat avenin challenge as previously reported.[7]

Single-bolus oat avenin challenge generated elevations in serum IL-2 in 11 of 29 (38%) participants across all doses of avenin (Figure 2A). This equated to 7 of 25 (28%) participants who had not previously been enrolled in oats challenge studies with no known clinical oats intolerance. Overall, four CD patients were positive for IL-2 after 0.5g avenin (equivalent to a 50 g serving of whole oats), and six CD patients were positive for IL-2 at the 4g dose. The

proportion of CD patients having a positive IL-2 increased with avenin dose, from 3/29 with 0.1g avenin to 6/29 with 4g avenin. Elevated IL-2 (> 2pg/mL) after avenin was associated with age over 50 years (Chi-square, p = 0.037) but no relationship was seen with age at CD diagnosis or the presence of HLA-DQ2.5 homozygosity.

Of the four participants with prior evidence of avenin-specific T cell responses (Figure 2A; blue = "oats responders"), 3/4 had IL-2 responses at the initial lowest dose consumed, one of which was highly symptomatic at this dose and did not continue with dose-escalation (#CD27). The two that continued to 0.5g had a high IL-2 fold-change at this dose and one was also highly symptomatic and was unable to continue with dose-escalation. All three undertook a four-week wash out period and then conducted a 0.05g avenin challenge (equivalent to approximately 5g whole oats). All three patients were positive for IL-2 at this dose (Figure 2A). The fourth patient (#CD28) became IL-2 positive at 1g avenin and was also positive at 4g avenin, but did not continue to the highest dose due to COVID-19 lockdown restrictions halting research.

Adverse gastrointestinal and extraintestinal (lethargy and headache) symptoms were recorded in 17 of 29 (59%) participants across all doses of avenin (Supplementary Table 2). Two participants (#CD1 and #CD2) additionally reported brain fog. While symptoms were generally mild one participant (#CD27) experienced severe vomiting. Symptoms were qualitatively similar to that seen after wheat gluten ingestion in CD with a predominance of gastrointestinal symptoms, with similar onset (within 4 hours) and resolution.[22] Three participants (#CD6, #CD7, #CD10) reported symptom onset between 6-24 hours.

At the highest avenin dose ingested, symptom severity was generally greater in participants with higher serum IL-2 (Figure 2B), supporting the notion that these symptoms were linked to T cell activation and were CD specific, differentiating them from other causes such as functional symptoms. Collectively, the findings indicate that oat avenin can trigger immune and symptom responses in many CD patients including at doses comparable to a standard serving size of oats.

Six-week oat avenin ingestion induced transient activation of avenin-specific T cells

Based on the findings from the single-dose study, seven IL-2 positive responders and one IL-2 non-responder (negative control) were selected to undertake a 6-week avenin challenge

(Table 2 and Figure 1). The dose of avenin ingested was the highest tolerated dose in the initial dose-ranging study that induced a significant IL-2 elevation. Therefore, two participants consumed low dose avenin of 0.05g - 0.5g and the remaining six ingested 4-6g avenin daily, equivalent to approximately 8-12 bowls of porridge daily. A treated CD participant (#CD33 W) included as a positive control consumed vital wheat gluten (10g) daily for 6 weeks and demonstrated classical duodenal histologic and serologic changes of CD at the end of this period. Four patients consumed avenin for the full 6 weeks and one participant consumed avenin for 5 weeks (38 days), stopping due to a COVID-19 lockdown, and was therefore included in the analyses. Two participants (#CD14 and #CD16) had clinical samples for histology but not research samples collected due to a COVID-19 lockdown limiting research. The known highly sensitive participant (#CD27) developed initial vomiting and later dropped out after developing headache, lethargy, dry cough, sore throat and general aches suggestive of a viral illness (Table 2).

Table 2. Six-week avenin challenge participant details.

ID	Single dose avenin IL-2	Maximal symptoms reported in the first 4-days	Maximal symptoms reported in final week of challenge	Minimum tolerated avenin dose	Comments
CD28	Positive	Severe diarrhoea, vomiting, nausea; mild abdominal cramps/pain, bloating, flatulence, headaches	Mild flatulence, reflux symptoms	4g	Past oat responder*
CD12	Positive	Moderate nausea, headaches and lethargy; mild constipation, flatulence	Moderate headache	4g	
CD22	Positive	Mild abdominal cramps/pain, bloating, flatulence, lethargy	Mild lethargy	6g	
CD14	Positive	Mild abdominal pain, bloating, diarrhoea	Mild headache	0.5g	Past oat responder* ; stopped day 38; clinical samples for histology only at follow-up
CD16	Positive	Mild flatulence	Mild flatulence	6g	Clinical samples for

					histology only at follow-up
CD9	Positive	Moderate constipation, diarrhoea, flatulence, headaches, lethargy; mild bloating	Moderate flatulence, headaches, lethargy; mild constipation and bloating	6g	Did not complete due to COVID-19 lockdown
CD27	Positive	Severe nausea, vomiting; mild bloating and lethargy	Severe lethargy, dry cough, sore throat; moderate headache	0.05g	Past oat responder*; Developed vomiting. Dropped out due to viral-like symptoms
CD1	Negative	Asymptomatic	Asymptomatic	6g	Did not complete due to COVID-19 lockdown
* As reported in Hardy et al, J Autoimmunity 2015[7]					

IL-2 measurements 0h and 4h post challenge prior to starting the 6-week challenge increased in all seven previous IL-2 responders but not in the previous IL-2 negative participant (Figure 3A). Similarly, in the wheat control patient, IL-2 increased 4 hours after wheat vital gluten ingestion (Figure 3A; red). At the end of the 5-6 week challenge 4-hour serum IL-2 levels after the final dose of avenin or wheat were only raised in the wheat control (Figure 3B; #CD33 W), and levels were lower than those observed at the start of the challenge. 92-plex proteomic screening showed that for the three CD patients with matched samples, avenin did not induce inflammatory cytokines at baseline or at follow-up (Figure 3C). Substantial increases in inflammatory cytokines were observed in the baseline sample from the wheat control, which were lower at the follow-up timepoint after the final dose of wheat (Figure 3C). Of the patients with no matched follow-up samples, there was one "oats sensitive" participant who had previously reacted at 0.1 g avenin with vomiting that had multiple raised inflammatory cytokines following their tolerated dose of 0.05g avenin (Figure 3D; #CD27). The inflammatory profile was comparable to the baseline from the wheat control (#CD33), although showed higher elevations in IL-17C and IL-6 and a lower elevation in IFN-γ (Figure 3C-D). All remaining avenin challenged participants, including two considered “oats

sensitive" (#CD14 and #CD28), did not show the raised inflammatory profile at baseline (Figure 3C-D).

Next, avenin-specific T cell frequency and activation status was examined by employing an MHC class II tetramer containing the immunodominant oat avenin epitope DQ2.5-ave-1c. There was a significant increase in frequency of tetramer+integrin β7+ T effector memory (Tem; CD45RA-CD62L-) CD4+ T cells in the blood on day 6 of the 6 week challenge (Figure 4A). Further, as has been reported for gluten-specific T cells after wheat gluten challenge, there was a significant increase in CD38-expressing tetramer+integrin β7+CD4+ Tem that was not observed in the tetramer- CD4+ Tem (Figure 4B-C). However, the percentage of CD38 expressing cells induced by avenin was generally lower than that observed following wheat gluten.[17]

In the tetramer+ population, the ratio of Tem to Tnaïve (CD45RA+CD62L+) increased on day 6 in most patients, but this was not significant and did not change significantly after 6 weeks in the 3 individuals tested (Figure 4D). No changes were observed in the ratio of Tem to Tnaïve in the tetramer- population (Figure 4E). The ratios observed in the avenin tetramer+ population were lower than those previously seen with alpha-gliadin peptide tetramer+ T cells, which were over one in the majority of treated and untreated CD patients.[23] Despite the increase in avenin tetramer+ CD4+ T cells on day 6, the number of circulating tetramer+ cells generally returned to baseline levels by the 6 week mark (Figure 4E-F).
The frequency of tetramer+ CD4+ T cells in the intestinal biopsies did not significantly increase or decrease following 6 weeks of avenin (Figure 4G). Tetramer+ CD4+ T cells in the intestine were CD38+ indicating activation, LAG-3- indicating lack of exhaustion, and did not display a CD25+CD127dim Treg phenotype (Supplementary Figure 1A-B).

Six-week oat avenin ingestion was clinically well tolerated
Purified avenin was well tolerated over the 6-week period. For the CD participants who reported initial adverse gastrointestinal symptoms these were generally transient and resolved during the period of avenin ingestion and did not cause the participant to prematurely stop (Table 2).

There was no significant duodenal mucosal deterioration after the 6-week avenin challenge ($p = 0.5$). The villous height: crypt depth (Vh:Cd) ratio from 1st and 2nd part of the duodenum was generally stable or increased (Figure 5A-B). Similarly, the IEL count from the 1st and 2nd part of the duodenum did not significantly increase after avenin (Figure 5C-D; $p > 0.99$ D2 and D1). In #CD22 A there was increased IEL that was not statistically significant. Following wheat challenge the Vh:Cd fell and IEL count increased (Figure 5A-D; red line). After the 6-week avenin challenge there was no rise in serum tTG-IgA or DGP-IgG in any participant (Figure 5E-F) and serum avenin protein-specific IgA was not elevated (Figure 5G). After the 6-week wheat gluten challenge the tTG-IgA seroconverted to positive (Figure E; red line).

Unfortunately, due to a COVID-19 pandemic restriction serology and gastroscopy was unable to be performed on participant #CD27 A, the super-sensitive patient with vomiting and the pro-inflammatory cytokine profile (Figure 3D).

Extended (three month) oat ingestion did not stimulate immune or clinical effects

To extend understanding of the clinical significance of avenin-specific IL-2 responsiveness, a 3-month gluten contamination-free whole oat feeding study (50 g daily) was undertaken. Eight CD participants that were IL-2 positive responders (n=3) and non-IL-2 responders (n=5) to single-bolus avenin challenge or 50g oats challenge participated (Supplementary Table 1). In three non-IL-2 responders with matched serum IL-2 at baseline and 3-month follow-up (#CD30, #CD31, #CD32) there was no increase in IL-2 at either timepoint (Figure 6A-B). Olink assessment was also undertaken in these matched samples and showed no increase in other inflammatory cytokine levels at 4h at baseline or follow-up (Supplementary Figure 2). Mirroring the 6-week avenin challenge results, in all participants tested there were no changes in avenin tetramer+ CD4+ T cell frequency in the blood or duodenum after 3 months (Figure 6C-D), and duodenal histology and CD serology did not deteriorate (Figure 6E-H).

DISCUSSION

To our knowledge, this is the first controlled oat feeding study in CD that reconciles the knowledge gap between the safety of oats from clinical studies and the indisputable evidence of avenin-specific T-cell responses in some CD patients. A major driver for this research was to resolve uncertainty about oats safety in CD as there is a need to improve the nutritional quality of the GFD. Population studies in CD show elevated rates of metabolic and

cardiovascular disease[24-26] and oats offer an excellent source of resistant starch such as beta-glucan and fibre, have a low glycaemic index and can reduce cardiovascular risk factors.[27, 28] Long-term oat ingestion has also been associated with improved quality of life in CD.[29] Our findings show that acute immune and symptom responses do occur to purified oat avenin in a dose-dependent manner in CD. While a third of CD patients can react acutely, this was generally not accompanied by any concerning histologic, symptomatic or serologic deterioration with prolonged exposure. In one patient (3% of cases), severe symptomatic and immune activation by oats avenin was accompanied by pro-inflammatory signals qualitatively and quantitatively similar to that induced by wheat, suggesting that a small number of CD patients are super-sensitive to avenin.

There are several strengths to this study. Firstly, we developed and utilised a novel approach to purify oats avenin to a level suitable for controlled high-dose feeding studies.[19] By using oat avenin, we could provide doses not possible with whole oats, as 6g avenin is equivalent to 12 bowls of oat porridge with a serving size of 50g. This is important, as it is clear there is a continuum of avenin responsiveness, and a standard serve of oats may provide insufficient avenin to induce consistent immune or clinical responses. Indeed, Moulton and colleagues back in 1958,[30] noted that to truly address the safety of oats the use of oats protein is required, however the technical capacity to purify avenin eluded them. By using low FODMAP avenin, we avoided the issues of high fibre load seen in whole oats and FODMAPs, both of which can confound symptom interpretation by triggering non-specific or functional gut symptoms.

Secondly, we utilised the IL-2 biomarker to assess gluten-induced T cell activation and symptoms, an approach that is more sensitive to low amounts of ingested gluten than T cell ELISpot, serology, duodenal histology and flow cytometry.[15] Supporting its higher sensitivity, we detected circulating IL-2 in a larger proportion of patients after oat ingestion (38% overall and 28% when examining those with unknown oat IL-2 responses) compared to utilising the IFN-γ ELISpot performed on 3-day oats challenged PBMCs (8%).[7] We used a more conservative cut-off than that previously published for wheat challenge, but if we were to apply that cut-off of 2.0, then the proportion of IL-2 responders at 6g avenin increased to 52%. This confirms at a biological level, half of CD patients harbour T cells that can recognise oat avenin, even if the doses ingested in the real world are unlikely to lead to harm.

Nevertheless, super-sensitivity does occur, as we noted IL-2 responses even to 50 mg avenin, equivalent to a 1/10th of a standard serve of porridge. The correlation between oat avenin induced gastrointestinal symptom severity and circulating IL-2 closely resembles our observations after wheat gluten challenge, where greater symptoms are noted when IL-2 is greater than 5 pg/mL.[13] A strength of using the IL-2 biomarker is that it confirms which symptoms reported by participants following oats are CD-related and not caused by functional gut upset, excess fibre or a nocebo effect.

The lack of enteropathy caused by oats compared to wheat remains an important unanswered question. Acute immune responses to wheat show a broad, sustained and significant cytokine response that is both T-cell and non-T cell derived that triggers a poorly defined cascade leading to tissue injury. Activation of CD103+ γδ and CD8+ intraepithelial effector T cells is important in wheat-induced CD enteropathy.[2, 31, 32] After oats, a comparable pro-inflammatory cytokine response is uncommon, suggesting a threshold to activate downstream effector mechanisms to cause enteropathy is not reached. Lower avenin immunogenicity could be explained by the lower content of immunogenic epitopes in avenin, their susceptibility to digestion by intestinal proteases, and their poor binding ability to HLA-DQ2.5.[7, 33] Furthermore, avenin-specific T cell activation (IL-2 production and CD38 expression) and frequency was not sustained with ongoing oat avenin ingestion, which is in contrast with wheat where pro-inflammatory cytokines after exposure remained elevated, albeit at a reduced level from baseline. Collectively, our data indicates that oat avenin can induce immune effects, but the dose of avenin-specific T cell epitopes are generally insufficient to trigger clinical effects except in the super-sensitive individuals.

Further potential explanations for the relative safety of oats exist. We noted that avenin did not drive a CD-relevant B cell response, as tTG-IgA, DGP-IgG levels and avenin-specific IgA did not rise despite sustained oat intake. This supports the lack of value in using CD serology to assess oat safety and also suggests potential differences in antigen uptake, processing, and presentation compared to gluten. tTG:gluten peptide complexes likely play a role in efficient peptide presentation[34, 35] but there is no evidence tTG:avenin peptide complexes form. Future studies should explore the role of other cell types, such as CD8 T cells, and examine duodenal tissue responses after avenin ingestion at a proteomic and

transcriptomic level to determine if novel pathways of immune tolerance or exhaustion are at play.

The molecular basis for super-sensitivity to oats is unknown but variable T cell sensitivity to wheat gluten in CD is well reported.[36] In a large CD cohort after controlled wheat challenge, we showed HLA gene-dose effect, older age and older age at diagnosis were associated with higher IL-2 elevations after wheat gluten. In this study, the highest elevations in IL-2 (> 2pg/mL) after single-bolus avenin ingestion was significantly associated with older participant age (> 50 years) but not age at diagnosis or HLA-DQ2.5 homozygosity. Future studies should look at whether these highly-sensitive T cells express high affinity oats avenin or cross-reactive barley-specific T cell receptors or are capable of inducing B cell responses. The role of barley ingestion in priming these avenin-specific T cells should also be assessed. Interestingly, regular oats ingestion in CD has been associated with dampened symptoms after barley ingestion, raising the possibility that tolerance to these oats peptides can be transferable to the related hordein sequences.[37]

There are limitations to our study. Firstly, whilst larger participant numbers would have enhanced generalisability, we had a limited source of avenin protein and a restrictive COVID-19 lockdown meant recruitment and follow-up was challenging. Unfortunately, the super-sensitive CD participant with the vomiting and pro-inflammatory cytokine response to avenin did not have follow-up histology due to the development of a viral illness. Secondly, it is possible the doses utilised for extended avenin challenge of 4 - 6g may be insufficient to induce enteropathy in all CD patients. However, studies employing 3g wheat gluten daily for 6 weeks generally show consistent histoconversion.[38, 39] Finally, while we did not show a clear relationship between tetramer+ frequency of avenin-specific T cells and responsiveness to avenin ingestion, we only measured a single avenin epitope and other avenin epitope-specific T cells may contribute.

Our findings have translational implications. Firstly, we definitively confirmed that oat avenin can induce adverse symptoms and immune activation in CD, validating the lived experience of some CD patients, but that in most cases extended ingestion is safely tolerated. Nevertheless, 3% of CD patients appear super-sensitive and develop severe symptoms like vomiting and pro-inflammatory response mimicking that induced by wheat. The CD field is increasingly acknowledging the importance of the systemic immune response to gluten over

enteropathy alone in CD, as evidenced by application of the IL-2 assay in drug development and revised diagnostic guidelines in children that focus on antibody levels rather than enteropathy.[3] The use of novel approaches such as in vivo or in vitro IL-2 release to oat avenin to identify the super-sensitive CD patient should be explored in future studies. At a food regulatory level, it remains important that food labelling clearly defines the presence of oats and allows CD patients who wish to exclude oats be able to do so. At a clinical level, the possibility of super-sensitivity to oats underscores the importance of clinical follow-up in CD.[40, 41]

Acknowledgments The authors would like to thank all the volunteers who participated in this study and Coeliac Australia and Coeliac UK for funding support. JT-D was supported by an NHMRC Investigator Grant (APP1176553). We thank Elizabeth Ly and Jane Muir (Monash University) for performing the FODMAP testing, Cathy Pizzey for help with study recruitment, sample collection, and examining the oats for grain contamination, and Kim and Danielle Rintoul for providing the gluten-contamination free oats.

Competing Interests MYH is a consultant for Takeda. JT-D has privately or via his institute been a consultant or advisory board member for Anatara, Anokion, Barinthus Biotherapeutics, Chugai Pharmaceuticals, Dr Falk, Forte Biosciences, IM Therapeutics, Janssen, Kallyope, Mozart Therapeutics, TEVA and Topas, has received research funding from Barinthus Biotherapeutics, Chugai Pharmaceuticals, Codexis, Immunic, Kallyope, Novoviah Pharmaceuticals, Topas and Tillotts Pharmaceuticals and received Honoraria from Takeda. He is an inventor on patents relating to the use of gluten peptides in coeliac disease diagnosis and treatment. All other authors have no conflicts to declare.

Ethics approval The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Melbourne Health and the Walter and Eliza Hall Institute (protocol codes 2020.162 and 20/21, respectively). Written informed consent was obtained from all participants prior to their involvement.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. Further data are available upon reasonable request.

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Jason Tye-Din: https://orcid.org/0000-0001-7687-9654

Figure legends:

Figure 1. Flow diagram of participants through the initial dose-response avenin feeding study, subsequent longer term (6 week and 3 month) feeding studies, and wheat challenged control. * As reported in Hardy et al, J Autoimmunity 2015.[7]

Figure 2. IL-2 is induced following pure avenin challenges. A) Serum from 29 participants was collected before (0h) and 4h after pure avenin challenge at escalating doses and with 4-week washout between each dose. IL-2 was measured in serum by mesoscale discovery S-plex. Coloured bars represent patients with IL-2 responses above the cut-off. Blue bars represent previous oats responders. * = $p < 0.05$ Wilcoxon signed rank test. B) Peak gastrointestinal symptom scores (asymptomatic, mild, moderate or severe) were graphed against IL-2 levels (pg/mL) recorded at the highest avenin dose for each patient (n=29; median ± IQR).

Figure 3. Pure avenin does not induce inflammatory responses in most patients. A) IL-2 was measured in serum samples before (0h) and 4h after pure avenin or wheat challenge at baseline prior to starting the 5-6 week challenge and B) on the last day of the 6 week challenge. Red bars represent the wheat control patient. Blue bars represent previous oats responders. * = $p < 0.05$ or NS = $p > 0.05$ Wilcoxon signed rank test comparing avenin challenged participants only. C) Matched baseline and follow-up and D) baseline only 0h and 4h serum following avenin challenge was tested using the Inflammation 96 O-link panel. Data shown are delta NPX (4h - 0h).

Figure 4. Avenin induced acute T cell responses. Samples were collected before the 6-week avenin challenge (baseline), on day 6 of the challenge, and after the challenge was completed (follow-up), DQ2.5-ave-1c-tetramer+ cells were enriched with magnetic cell separation and stained for phentotypic markers for flow cytometry. A) The number of tetramer+integrin b7 effector memory CD4 T cells. The percentage of CD38+ cells within the B) tetramer+ population and C) tetramer- population. D) The ratio of effector memory to naïve T cells

within the tetramer+ population (left) and tetramer- population (right). E) and F) Circulating Avenin tetramer+ integrin b7+ Teffector memory CD4+ T cells before and after avenin challenge (n=3). G) Avenin Tetramer + CD4+ T cells stained within lamina propria mononuclear cells (n=3). Blue bars represent previous oats responders. * = $p < 0.05$ or NS = $p > 0.05$ Wilcoxon signed rank test.

Figure 5. Clinical responses following long term avenin challenge. Villous height: crypt depth ratios for A) D2 and B) D1. Intraepithelial lymphocyte counts for C) D2 and D) D1. E) tTG-IgA serology and F) DGP-IgG serology. G) Anti-avenin protein IgG responses in serum pre and post 6 weeks. Dotted lines indicate the cut-off (Vh:CD =2 and IEL = 25). Blue bars represent previous oats responders. Red bars represent the wheat control patient NS = $p > 0.05$ Wilcoxon signed rank test comparing avenin challenged participants only.

Figure 6. "Real-world" oats challenges do not induce immune responses or clinical deterioration after 3 months. IL-2 levels in serum at 0h and 4h at A) baseline prior to oats challenge and B) the last day of the 3month challenge. C) Circulating DQ2.5-ave-1c tetramer +integrin b7+ effector memory CD4+ T cells before and after 3month oats challenge. D) Avenin Tetramer+ CD4+ T cells stained within lamina propria mononuclear cells. E) Villous height: crypt depth ratios for D2. F) Intraepithelial lymphocyte counts for D2. G) tTG-IgA serology and H) DGP-IgG serology. Dotted lines indicate the cut-off (Vh:CD =2 and IEL = 25). Blue bars represent previous oats responders. NS = $p > 0.05$ Wilcoxon signed rank test.

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Figure 1

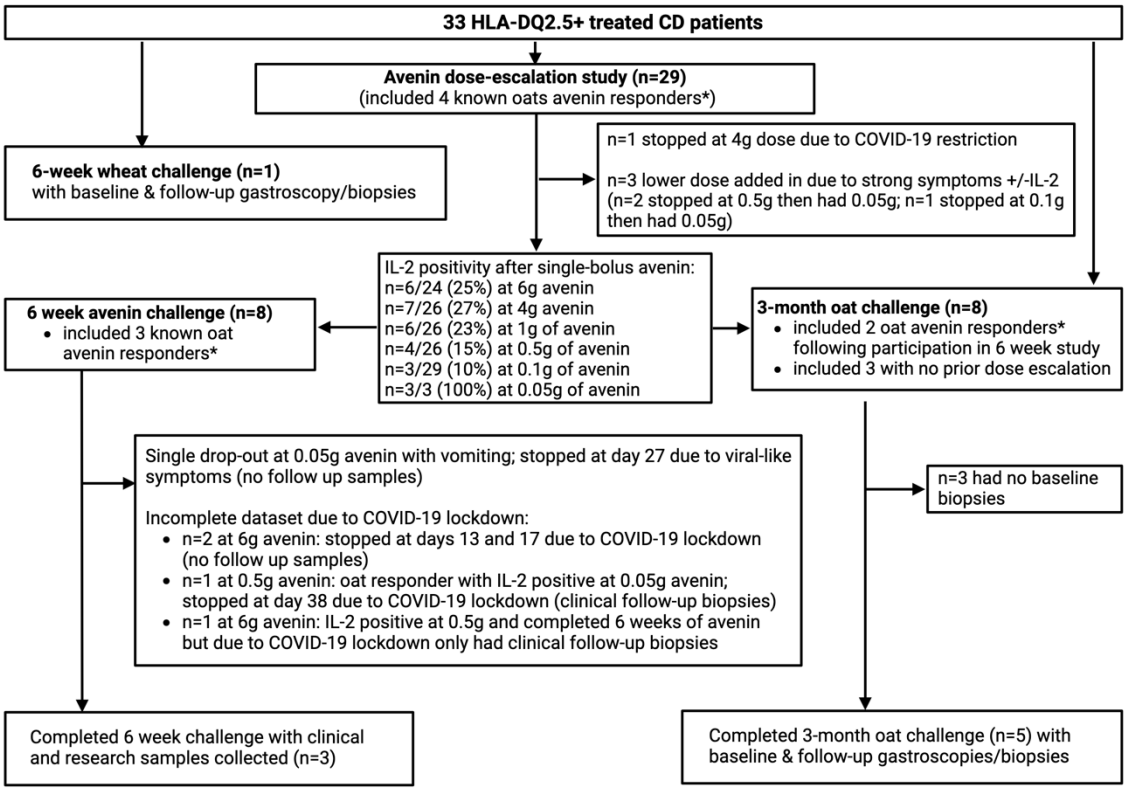
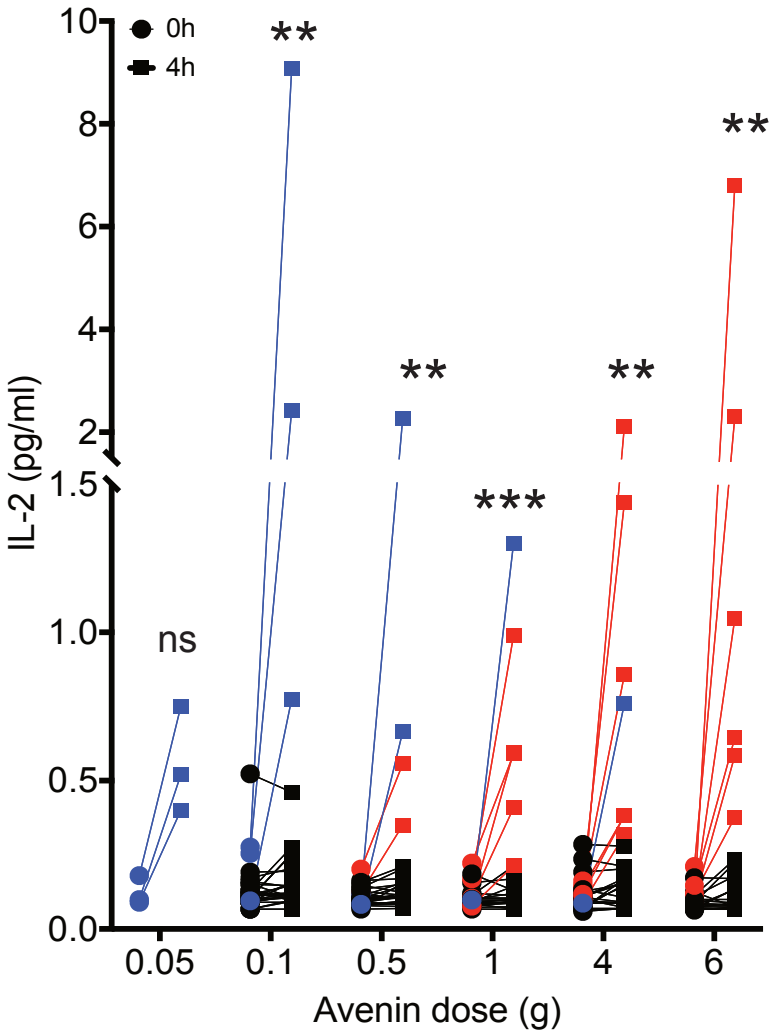

33 HLA-DQ2.5+ treated CD patients
Avenin dose-escalation study (n=29)
(included 4 known oats avenin responders*)
6-week wheat challenge (n=1)
with baseline & follow-up gastroscopy/biopsies
n=1 stopped at 4g dose due to COVID-19 restriction
n=3 lower dose added in due to strong symptoms +/-IL-2
(n=2 stopped at 0.5g then had 0.05g; n=1 stopped at 0.1g then had 0.05g)
IL-2 positivity after single-bolus avenin:
n=6/24 (25%) at 6g avenin
n=7/26 (27%) at 4g avenin
n=6/26 (23%) at 1g of avenin
n=4/26 (15%) at 0.5g of avenin
n=3/29 (10%) at 0.1g of avenin
n=3/3 (100%) at 0.05g of avenin
6 week avenin challenge (n=8)
included 3 known oat avenin responders*
3-month oat challenge (n=8)
included 2 oat avenin responders* following participation in 6 week study
included 3 with no prior dose escalation
Single drop-out at 0.05g avenin with vomiting; stopped at day 27 due to viral-like symptoms (no follow up samples)
Incomplete dataset due to COVID-19 lockdown:
n=2 at 6g avenin: stopped at days 13 and 17 due to COVID-19 lockdown (no follow up samples)
n=1 at 0.5g avenin: oat responder with IL-2 positive at 0.05g avenin; stopped at day 38 due to COVID-19 lockdown (clinical follow-up biopsies)
n=1 at 6g avenin: IL-2 positive at 0.5g and completed 6 weeks of avenin but due to COVID-19 lockdown only had clinical follow-up biopsies
n=3 had no baseline biopsies
Completed 6 week challenge with clinical and research samples collected (n=3)
Completed 3-month oat challenge (n=5) with baseline & follow-up gastroscopies/biopsies

Figure 2

A


0h
4h
IL-2 (pg/ml)
10
8
6
4
2
1.5
1.0
0.5
0.0
ns
**
**
***
**
**
0.05
0.1
0.5
1
4
6
Avenin dose (g)

B

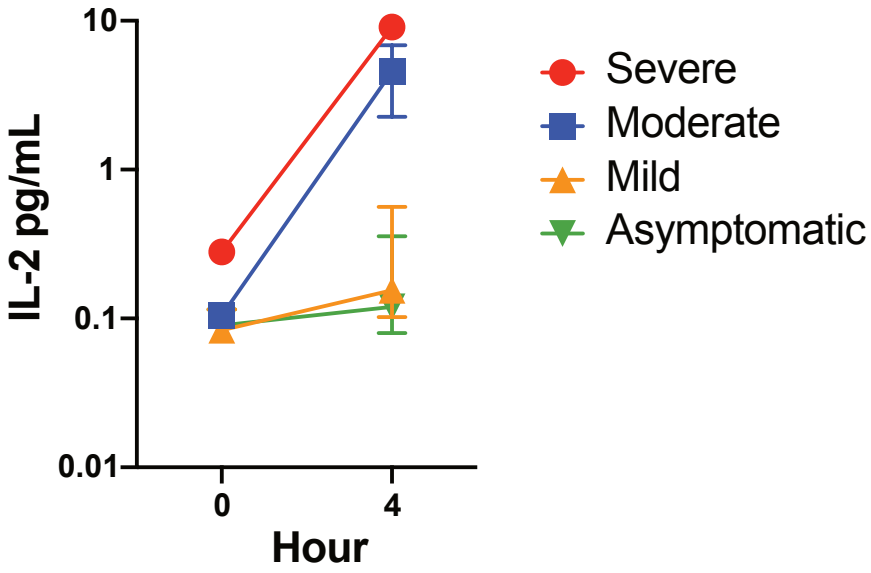
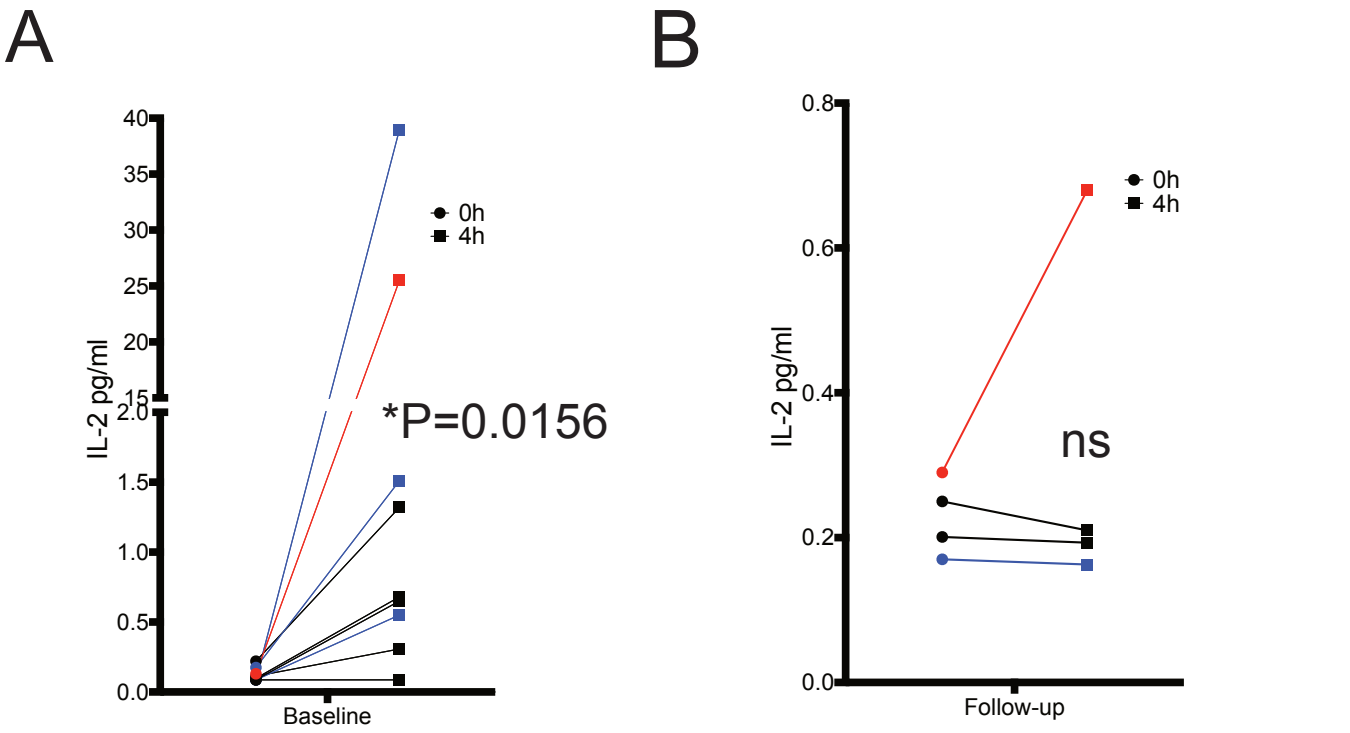
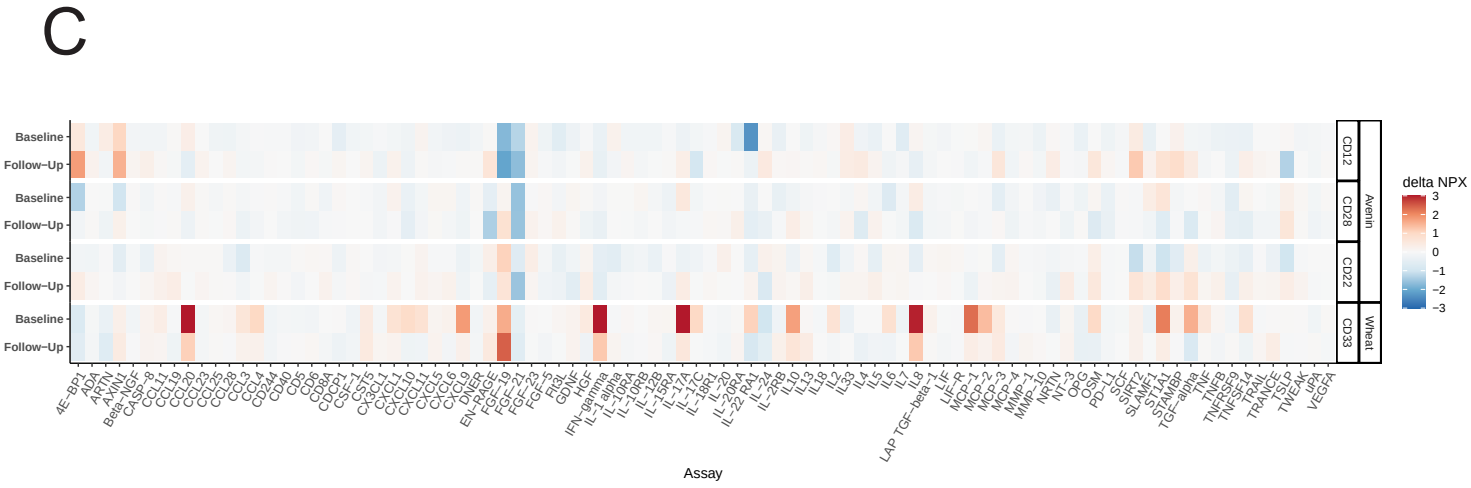

IL-2 pg/mL
10
1
0.1
0.01
0
4
Hour
Severe
Moderate
Mild
Asymptomatic

Figure 3


A
B
IL-2 pg/ml
0h
4h
*P=0.0156
Baseline
ns
Follow-up


C
Baseline
Follow-Up
CD12
CD28
CD22
CD33
Avenin
Wheat
delta NPX
Assay

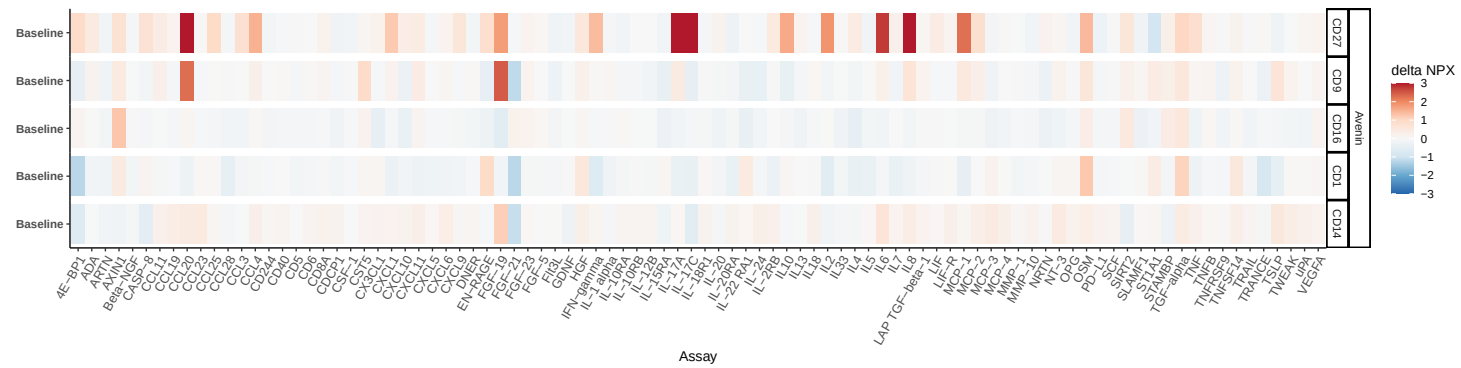

D
Baseline
CD27
CD9
CD16
CD1
CD14
Avenin
delta NPX
Assay

Figure 4

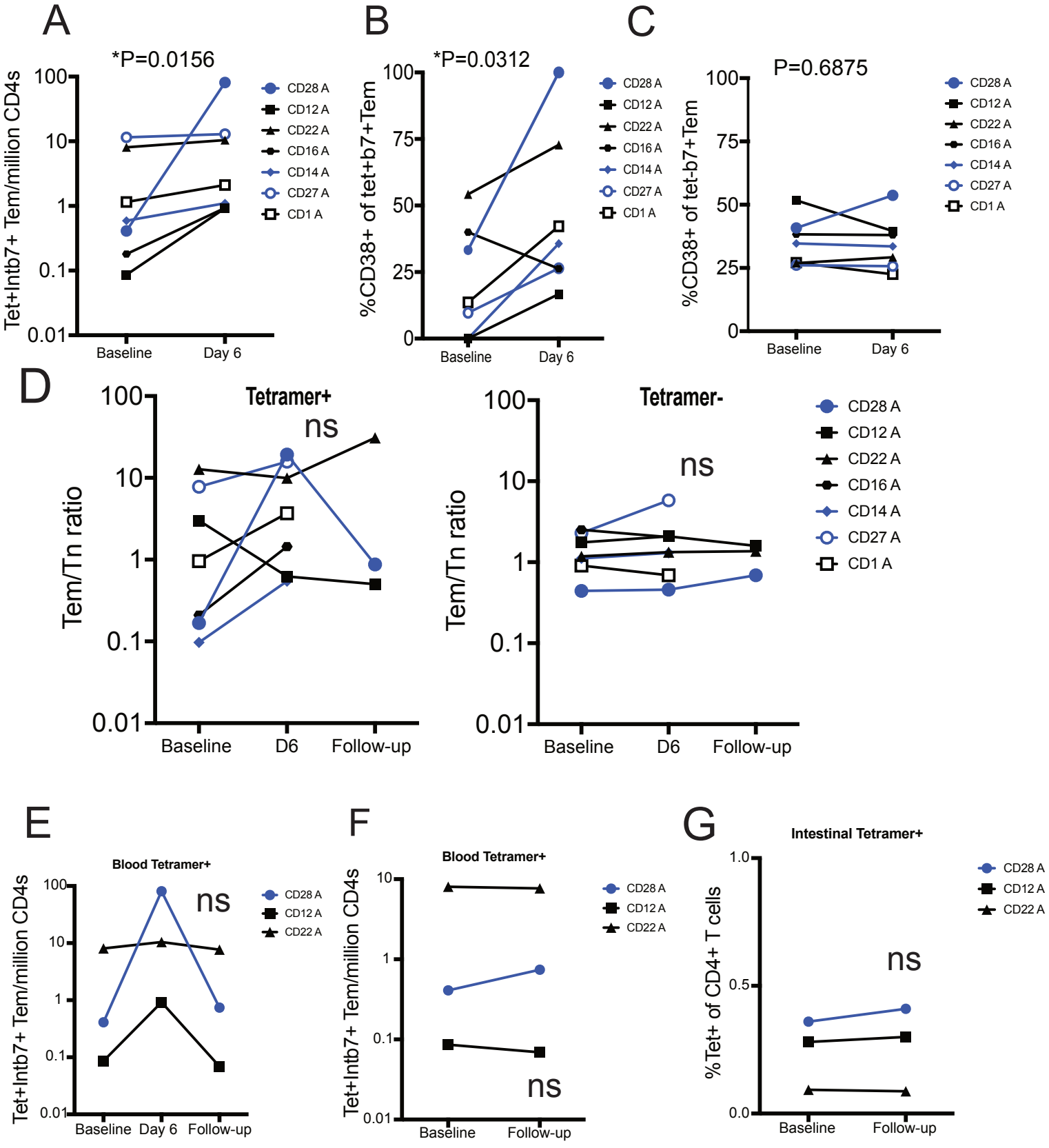
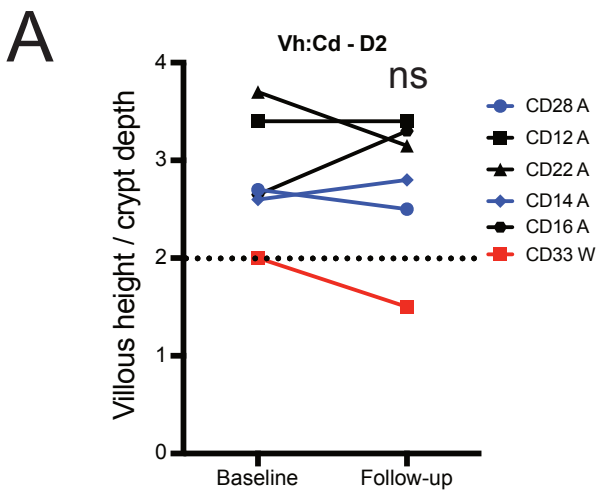
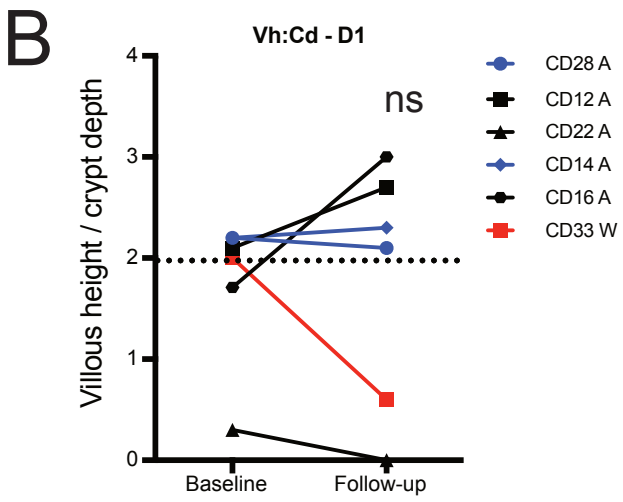
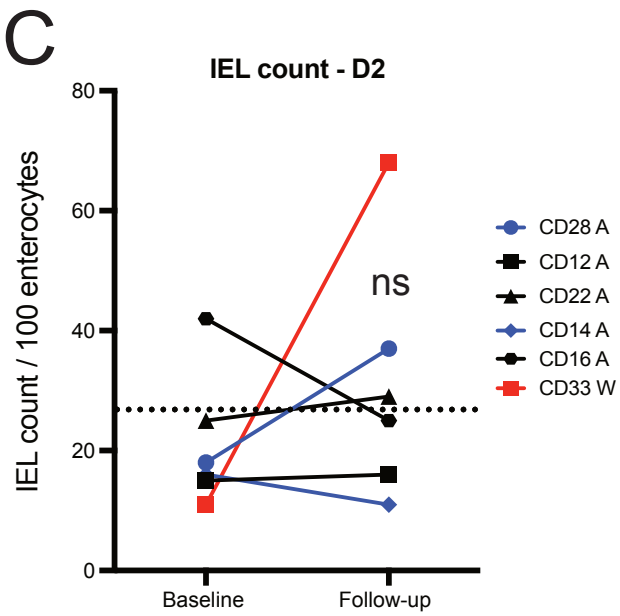
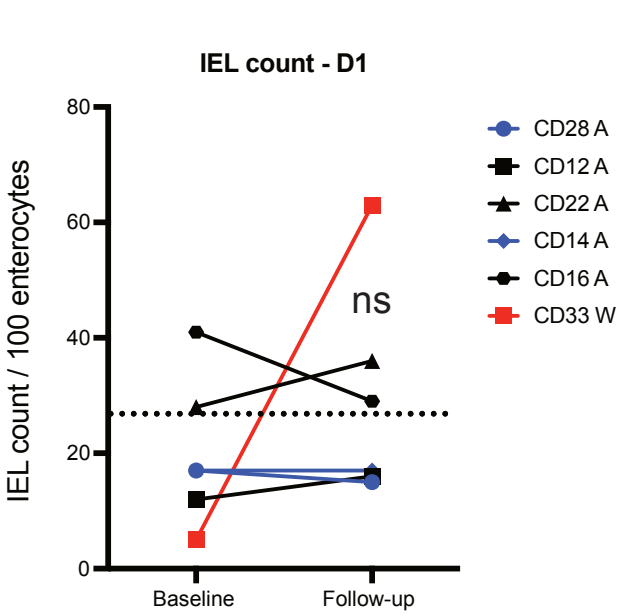

A
*P=0.0156
Tet+Intb7+ Tem/million CD4s
100
10
1
0.1
0.01
Baseline
Day 6
CD28 A
CD12 A
CD22 A
CD16 A
CD14 A
CD27 A
CD1 A
B
*P=0.0312
%CD38+ of tet+b7+Tem
100
75
50
25
0
Baseline
Day 6
CD28 A
CD12 A
CD22 A
CD16 A
CD14 A
CD27 A
CD1 A
C
P=0.6875
%CD38+ of tet-b7+Tem
100
75
50
25
0
Baseline
Day 6
CD28 A
CD12 A
CD22 A
CD16 A
CD14 A
CD27 A
CD1 A
D
Tetramer+
ns
Tem/Tn ratio
100
10
1
0.1
0.01
Baseline
D6
Follow-up
Tetramer-
ns
Tem/Tn ratio
100
10
1
0.1
0.01
Baseline
D6
Follow-up
CD28 A
CD12 A
CD22 A
CD16 A
CD14 A
CD27 A
CD1 A
E
Blood Tetramer+
ns
Tet+Intb7+ Tem/million CD4s
100
10
1
0.1
0.01
Baseline
Day 6
Follow-up
CD28 A
CD12 A
CD22 A
F
Blood Tetramer+
Tet+Intb7+ Tem/million CD4s
10
1
0.1
0.01
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A
G
Intestinal Tetramer+
%Tet+ of CD4+ T cells
1.0
0.5
0.0
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A

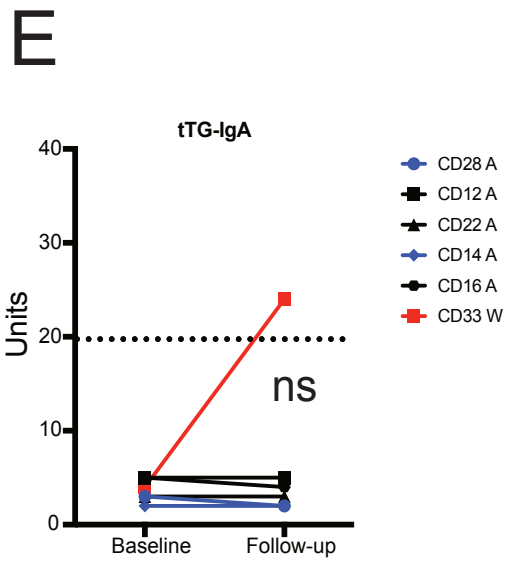
Figure 5

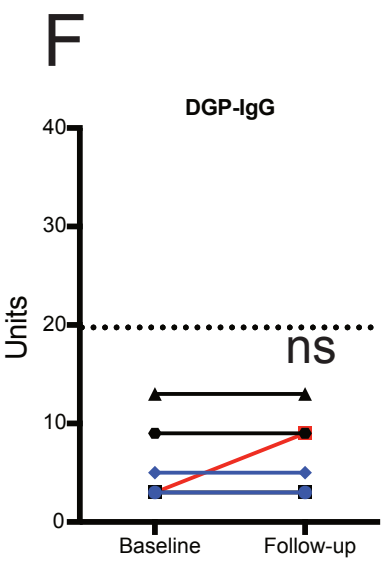

A
Vh:Cd - D2
Villous height / crypt depth
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A
CD14 A
CD16 A
CD33 W


B
Vh:Cd - D1
Villous height / crypt depth
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A
CD14 A
CD16 A
CD33 W


C
IEL count - D2
IEL count / 100 enterocytes
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A
CD14 A
CD16 A
CD33 W


D
IEL count - D1
IEL count / 100 enterocytes
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A
CD14 A
CD16 A
CD33 W


E
tTG-IgA
Units
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A
CD14 A
CD16 A
CD33 W


F
DGP-IgG
Units
ns
Baseline
Follow-up

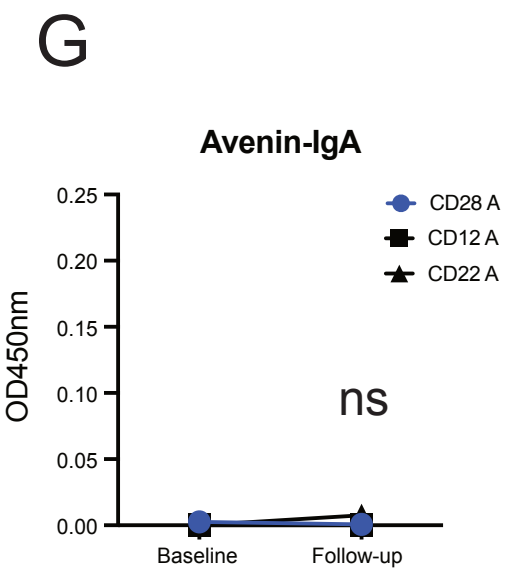
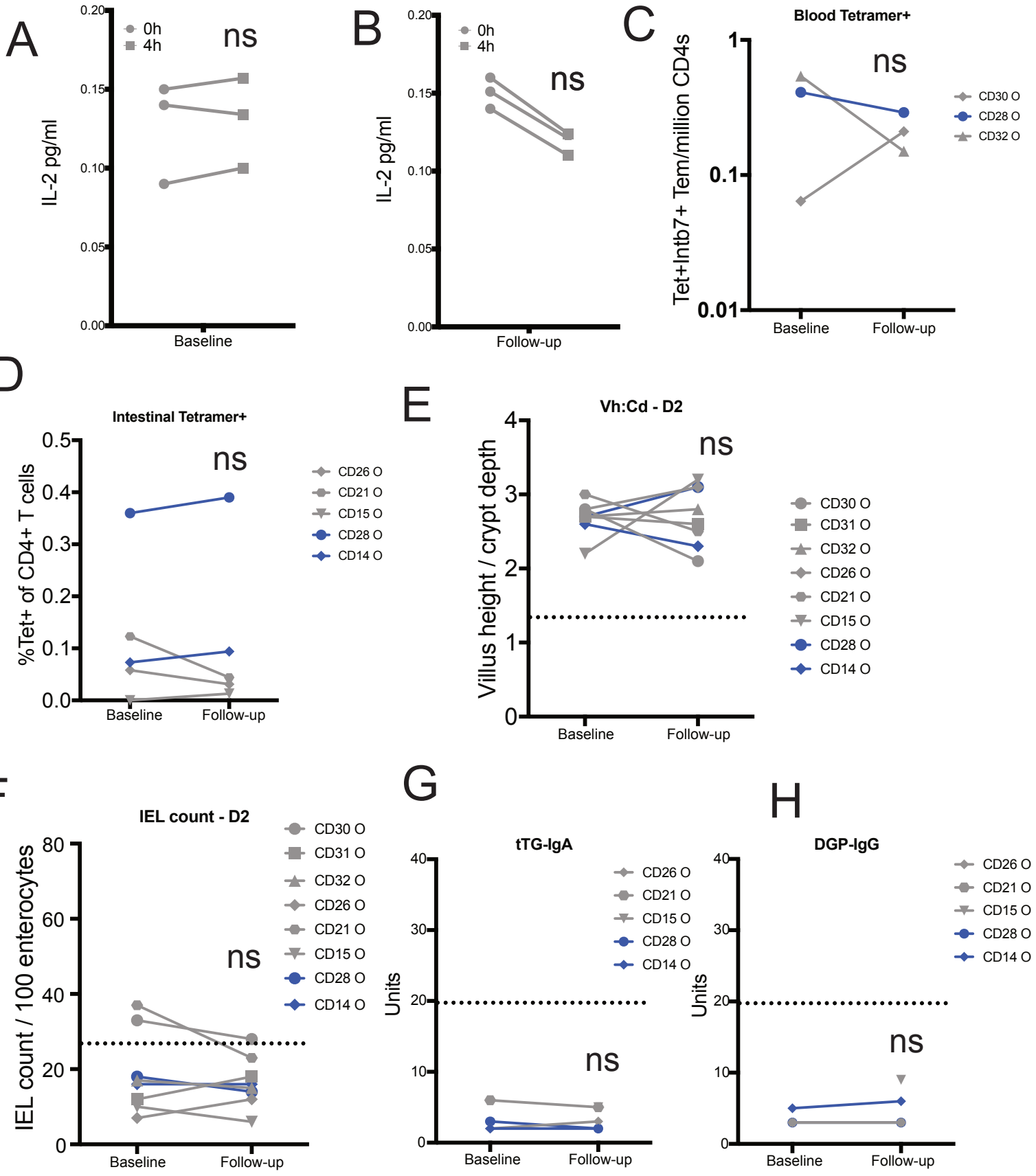

G
Avenin-IgA
OD450nm
ns
Baseline
Follow-up
CD28 A
CD12 A
CD22 A

Figure 6


A
0h
4h
ns
IL-2 pg/ml
0.20
0.15
0.10
0.05
0.00
Baseline
B
0h
4h
ns
IL-2 pg/ml
0.20
0.15
0.10
0.05
0.00
Follow-up
C
Blood Tetramer+
ns
Tet+Intb7+ Tem/million CD4s
1
0.1
0.01
Baseline
Follow-up
CD30 O
CD28 O
CD32 O
D
Intestinal Tetramer+
ns
%Tet+ of CD4+ T cells
0.5
0.4
0.3
0.2
0.1
0.0
Baseline
Follow-up
CD26 O
CD21 O
CD15 O
CD28 O
CD14 O
E
Vh:Cd - D2
ns
Villus height / crypt depth
4
3
2
1
0
Baseline
Follow-up
CD30 O
CD31 O
CD32 O
CD26 O
CD21 O
CD15 O
CD28 O
CD14 O
F
IEL count - D2
ns
IEL count / 100 enterocytes
80
60
40
20
0
Baseline
Follow-up
CD30 O
CD31 O
CD32 O
CD26 O
CD21 O
CD15 O
CD28 O
CD14 O
G
tTG-IgA
ns
Units
40
30
20
10
0
Baseline
Follow-up
CD26 O
CD21 O
CD15 O
CD28 O
CD14 O
H
DGP-IgG
ns
Units
40
30
20
10
0
Baseline
Follow-up
CD26 O
CD21 O
CD15 O
CD28 O
CD14 O