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ARTICLE

Restoration of impaired intestinal barrier function by the hydrolysed casein diet contributes to the prevention of type 1 diabetes in the diabetes-prone BioBreeding rat

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Abstract
Aims/hypothesis Impaired intestinal barrier function is observed in type 1 diabetes patients and animal models of the disease. Exposure to diabetogenic antigens from the intestinal milieu due to a compromised intestinal barrier is considered essential for induction of the autoimmune process leading to type 1 diabetes. Since a hydrolysed casein (HC) diet prevents autoimmune diabetes onset in diabetes-prone (DP)-BioBreeding (BB) rats, we studied the role of the HC diet on intestinal barrier function and, therefore, prevention of autoimmune diabetes onset in this animal model.
Methods DP-BB rats were fed the HC diet from weaning onwards and monitored for autoimmune diabetes development. Intestinal permeability was assessed in vivo by lactulose–mannitol test and ex vivo by measuring transepithelial electrical resistance (TEER). Levels of serum zonulin, a physiological tight junction modulator, were measured by ELISA. Ileal mRNA expression of *Myo9b*, *Cldn1*, *Cldn2* and *Ocln* (which encode the tight junction-related proteins myosin IXb, claudin-1, claudin-2 and occludin) and *Il-10, Tgf-ß* (also known as *Il10* and *Tgfb*, respectively, which encode regulatory cytokines) was analysed by quantitative PCR.
Results The HC diet reduced autoimmune diabetes by 50% in DP-BB rats. In DP-BB rats, prediabetic gut permeability negatively correlated with the moment of autoimmune diabetes onset. The improved intestinal barrier function that was induced by HC diet in DP-BB rats was visualised by decreasing lactulose:mannitol ratio, decreasing serum zonulin levels and increasing ileal TEER. The HC diet modified ileal mRNA expression of *Myo9b*, and *Cldn1* and *Cldn2*, but left *Ocln* expression unaltered.
Conclusions/interpretation Improved intestinal barrier function might be an important intermediate in the prevention of autoimmune diabetes by the HC diet in DP-BB rats. Effects on tight junctions, ileal cytokines and zonulin production might be important mechanisms for this effect.

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Keywords BB rat · Cytokines · Hydrolysed casein diet · Intestinal barrier · Tight junctions · Type 1 diabetes

Abbreviations

BB	BioBreeding
DP	Diabetes-prone
DR	Diabetes-resistant
HC	Hydrolysed casein
LA/MA	Lactulose–mannitol (test)
TEER	Transepithelial electrical resistance

Introduction

Both genetic and environmental factors are pivotal for the induction of type 1 diabetes. Possible environmental diabetogenic factors are dietary products and bacterial/viral infections [1, 2]. These diabetogenic triggers can induce an immune cascade, eventually leading to the autoimmune process that is typical of type 1 diabetes [1, 2].

We and others have shown that diet and gut microbiota are critical for autoimmune diabetes pathogenesis in the diabetes-prone (DP) BioBreeding (BB) rat model of type 1 diabetes [1, 3–8]. When fed a hydrolysed casein (HC) diet, only ±50% of DP-BB rats develop autoimmune diabetes [1, 3–6]. These studies also showed that besides lack of diabetogenic antigens in the food, skewing the (mucosal) immune response towards a less pathogenic Th2 phenotype and the induction of islet neogenesis are important mechanisms in the prevention of type 1 diabetes by the HC diet [1, 3–6].

There is growing evidence that a third factor, increased intestinal permeability, underlies the pathogenesis and/or perpetuation of at least some autoimmune disorders such as Crohn's disease and coeliac disease [9, 10]. Intriguingly, type 1 diabetes patients and animal models of type 1 diabetes show an impaired intestinal barrier function and signs of intestinal inflammation that precede the onset of type 1 diabetes [11–21]. In type 1 diabetes patients and DP-BB rats increased serum zonulin levels were found [13, 14]. Zonulin, the human analogue of Zot from *Vibrio cholerae*, is an intestinal physiological modulator of tight junctions. By binding to its receptor, this protein activates signalling pathways that cause opening of the tight junctions and therewith increased intestinal permeability [12]. Blocking the zonulin receptor by a specific antagonist led to reduced intestinal permeability in vitro and in vivo [14, 22, 23], and prevented the increase of intestinal permeability caused by bacterial agents or gliadin [23, 24]. Interestingly, zonulin antagonists restored the intestinal barrier function and subsequently delayed or prevented the development of autoimmune diabetes in DP-BB rats [14].

Food components and gut-derived bacteria can affect intestinal barrier integrity [12, 23, 24], but the role of the intestinal barrier in preventing autoimmune diabetes by HC diet has not been thoroughly investigated. It was shown that a HC diet improves the intestinal integrity of DP-BB rats [7, 16], but these studies did not investigate the effect of the HC diet on tight junctions, the zonulin system, gut microbiota and the relation with type 1 diabetes development.

We therefore investigated whether, besides the above-mentioned mechanisms, restoration of the impaired intestinal barrier function (with emphasis on tight junction proteins) also contributes to the prevention of autoimmune diabetes by a HC diet in DP-BB rats.

Methods

Animals Rats were derived from the Worcester DP-BB and diabetes-resistant (DR) BB strain, but were maintained and bred at our institutional Central Animal Facility under specific-pathogen-free and viral-antibody-free conditions [25]. The animals received humane care in compliance with the principles of laboratory animal care (NIH publication no. 85-23; revised 1985) and the Dutch law on experimental animal care. The university Ethical Board for Animal Studies approved all animal experiments reported in this study.

Dietary intervention protocol Two dietary intervention experiments were performed.

In the first, experiment 1, DP-BB rats were given the HC diet (n=18) or a standard diet (n=16) from weaning onwards and monitored for the development of autoimmune diabetes. At 65 days of age, just before DP-BB rats start to develop autoimmune diabetes, a lactulose–mannitol (LA/MA) assay as described by Meddings et al. [16] was performed to establish intestinal permeability in the small intestine. After subjection to the LA/MA test, the rats were monitored until 140 days of age. Animals were weighed three times per week. In case of weight loss or suspicion of diabetes, blood glucose was measured in tail vein blood using a glucose sensor (Reflolux S; Boehringer, Mannheim, Germany). When blood glucose exceeded 15 mmol/l (non-fasting), rats were considered diabetic and killed.

In the second intervention, experiment 2, another group of DP-BB rats was fed the HC diet (n=37) or the standard diet (n=33) from weaning onwards. At fixed time points (25, 35, 45, 55 and 65 days of age) rats were killed. Ileum tissue was collected to investigate the effect of the HC diet over time on the intestinal barrier function by measuring mRNA expression of tight junction proteins and the ileal transepithelial electrical resistance (TEER). The TEER was measured on ileal tissue obtained at 65 days of age. In

addition, ileal mRNA expression of the cytokines *Ifn-γ* (also known as *Ifng*), *Tnf-α* (also known as *Tnf*), *Il-10* (also known as *Il10*) and *Tgf-ß* (also known as *Tgfb1*) was measured. DR-BB rats of 21 to 50 days of age (n=5) or 51 to 70 days of age (n=25) were used as control rats.

Diet The standard plant-based diet used was a standard laboratory rodent diet (RMH-B diet; Arie Blok, Woerden, the Netherlands). The HC diet (TD99482; Harlan-Teklad Custom Research, Madison, WI, USA) is a modification of the AIN-93G diet and contained 200 g/kg HC (as source of amino acids), 3 g/kg L-cysteine, 509.8 g/kg corn starch, 120 g/kg sucrose, 70 g/kg soy-bean oil, 50 g/kg cellulose, 35 g/kg mineral mix, 10 g/kg vitamin mix, 2 g/kg choline bitartrate and 0.20 g/kg butylated hydroxyanisole antioxidant.

LA/MA test for measuring intestinal permeability in vivo The LA/MA test is a non-invasive technique to measure intestinal permeability in vivo. The sugar alcohol mannitol, which is a small molecule, permeates the intestinal mucosa via a transcellular pathway through the water-filled pores of the cell membrane, whereas the larger disaccharide molecule lactulose uses a paracellular route through the junctional complexes between adjacent enterocytes. An increased uptake of lactulose (associated with a high lactulose: mannitol ratio) indicates a damaged barrier. Therefore, a reduction of the intestinal permeability will lead to a lower lactulose uptake and a decreased lactulose:mannitol ratio in the urine. This test allowed us to investigate whether the rats with high intestinal permeability in the prediabetic phase would indeed develop autoimmune diabetes.

A LA/MA assay as described by Meddings et al. [16] was performed to establish intestinal permeability in the small intestine. Briefly, a stock solution was made containing 4 g mannitol and 6 g lactulose per 100 ml distilled water. Enough solution was made to give each rat 2 ml of the probe.

Rats were placed in stainless steel metabolism cages with wire bottoms to separate faeces from urine. Rats were denied access to water for 3 h, at which point they were allowed free access to water for the remainder of the experiment. Urine was collected for a total of 24 h, after which rats were returned to their normal cages. Urine volumes were measured and the urine composition was analysed by HPLC as described previously [16].

Final data were reported as a ratio of fractional excretions (lactulose:mannitol). Fractional excretion is defined as the fraction of the gavaged dose recovered in the urine sample.

Snapwell assay for measuring TEER An indicator of intestinal permeability, TEER was measured on ileum samples in vitro by snapwell assay as described by Watts et al. [14], with some minor modifications. A small sample (length ~50 mm) was taken from the ileum. During the time between killing of the animal and mounting in the snapwells (Corning, Schiphol-rijk, the Netherlands), the samples were kept in DMEM (with 4.5 g/l glucose) (Gibco, Breda, the Netherlands) at 4°C. Before mounting, the intestinal pieces were cleaned of faeces by gently pulling the intestine over a 1 ml pipette and then cut open longitudinally. The intestine was cleaned further by gently moving it, with the mucosal layer facing downwards, in a Petri-dish with approximately 1 ml medium. A piece was cut out, placed on a filter with the mucosal layer facing upwards, sandwiched between two disks and put in the snapwell insert. This insert was placed in a pre-warmed six-well plate containing 2 ml DMEM in each well. On top of the insert 450 μl DMEM was added. The tissue was mounted within 15 min after excision and the plates were incubated at 37°C. The TEER measurements were done using a Millipore Millicell-ERS meter (Millipore, Amsterdam, the Netherlands) at 30, 60, 90, 120 and 180 min after excision. The TEER shown in this paper was measured at 60 min after tissue excision.

Quantitative PCR of tight junction proteins and cytokines RNA was isolated from ileal tissue and expression of *Myo9b*, *Cldn1*, *Cldn2* and *Ocln* mRNA (encoding the tight-junction-related proteins myosin IXb, claudin-1, claudin-2 and occludin) and *Ifn-γ*, *Il-10*, *Tgf-ß* and *Tnf-α* mRNA (encoding cytokines) was analysed. To isolate RNA from the intestine, frozen tissue (±1 cm, stored at −80°C) was homogenised in 1 ml of TRI reagent (Sigma-Aldrich, Zwijndrecht, the Netherlands) and mRNA was isolated using the TRI reagent mRNA isolation method. The concentration of the isolated mRNA was determined using a nanodrop (ND-1000; Isogen, Maarsen, the Netherlands). Measurement was done at the 230 nm absorption spectrum for RNA. Isolated mRNA (5 μg) was converted to cDNA using a kit (SuperScript II Reverse Transcriptase kit; Invitrogen Life Technologies, Breda, the Netherlands). To measure differences in expression of genes for tight junction-related proteins, transcript levels of *Myo9b*, *Cldn1*, *Cldn2*, *Ocln* and the housekeeping gene hypoxanthine phosphoribosyl-transferase (*Hprt*) were quantified using real-time PCR (for primer sequences see Electronic supplementary material [ESM] Table 1). Real-time PCR analysis was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, the Netherlands) according to the manufacturer's instructions; detection was by iCycler iQ Real-Time PCR Detection System (Bio-Rad), using the following programme: 3 min 95°C, 40 cycles of 30 s at 95°C and of 30 s at 60°C, and of 10 s at 58°C, then 80 times an increase in temperature of 0.5°C to create a melting curve. Results were expressed as ratio of target gene, *Hprt*, according to a mathematical method described by Pfaffl et al. [26].

Zonulin ELISA Serum zonulin levels were analysed by the UM Center for Celiac Research at the University of Maryland

Medical Center. The ELISA was performed as described by Sapone et al. [15].

Statistical analysis Difference in survival was calculated by the logrank test for Kaplan–Meier survival curves. Differences in zonulin levels and lactulose:mannitol ratio were calculated using the Mann–Whitney *U* test. Differences in TEER and expression of tight junction proteins and cytokine levels were calculated by Kruskal–Wallis test followed by the Mann–Whitney *U* test to identify the difference between the groups. Correlations were tested for significance using the Spearman correlation method. A p value of <0.05 was considered significant.

Results

Relation between the level of prediabetic gut permeability and autoimmune diabetes development, and effects of the HC diet on intestinal barrier function in DP-BB rats As previously reported by our group and others [1, 3–7], the HC diet prevented and delayed the onset of autoimmune diabetes in DP-BB rats (Fig. 1a).

As seen in Fig. 1b, our results show that HC diet fed rats had a reduced urinary lactulose:mannitol ratio (i.e. less leaky gut). This shows that feeding the rats the HC diet improves intestinal integrity. Interestingly, in both groups, there was a significant correlation between the day of autoimmune diabetes onset and the prediabetic urinary lactulose:mannitol ratio at 65 days of age (Fig. 1c, d). In the rats on a standard diet this correlation was present when all the rats are included (diabetic and non-diabetic rats) (Fig. 1c) and when only the diabetic rats were analysed (Fig. 1d). In rats fed a HC diet, the correlation was only sizable when the diabetic rats were analysed. These results indicate a relation between prediabetic intestinal barrier function and the moment of diabetes outcome.

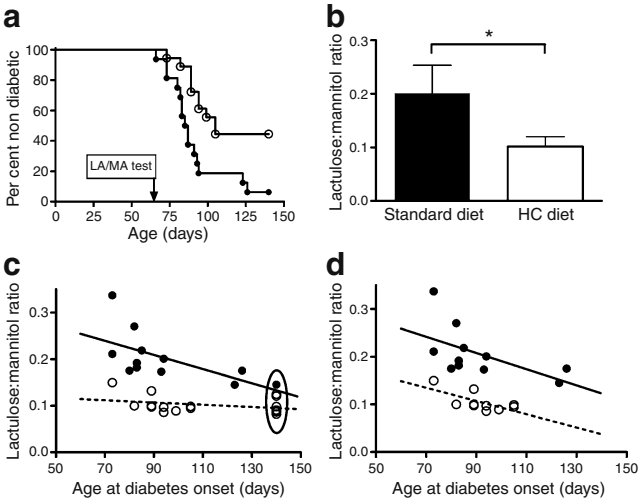

Fig. 1 HC diet, intestinal permeability and autoimmune diabetes development in DP-BB rats. **a** Kaplan–Meier survival curve showing the development of autoimmune diabetes in DP-BB rats fed a HC diet (white circles) or a standard diet (black circles). $p<0.05$, logrank test for Kaplan–Meier survival curves. **b** Urinary lactulose:mannitol ratio established at 65 days of age in DP-BB rats on the indicated diet. Standard diet, $n=12$ (three urinary samples not analysed owing to sampling problems; one rat excluded because it developed diabetes during LA/MA test); HC diet, $n=18$. *$p<0.05$ **c, d** Correlation between lactulose:mannitol ratio at 65 days of age and the age of diabetes onset in DP-BB rats fed the HC diet (white circles, dotted lines) or the standard diet (black circles, continuous lines). The data in **c** were analysed including the rats who did not develop type 1 diabetes before 140 days of age (marked by the oval). Dotted line: HC diet-fed DP-BB rats, $r=-0.4463$, p = NS; continuous line: standard diet-fed DP-BB rats, $r=-0.6819$, $p=0.0146$. Data in **d** were analysed excluding the rats who did not develop type 1 diabetes before 140 days of age. Dotted line: HC diet-fed DP-BB rats, $r=-0.7122$, $p=0.0209$; continuous line: standard diet-fed DP-BB rats, $r=-0.6041$, $p=0.0490$

The HC diet increases ileal TEER in DP-BB rats The ileal TEER of DP-BB rats fed the HC diet was measured ex vivo at 65 days of age using the microsnapwell assay described previously [14]. As control groups, DR-BB rats and DP-BB rats fed the standard diet were used. DP-BB rats on the standard diet had a lower TEER (i.e. increased intestinal permeability) than DR-BB rats on the same diet (Fig. 2), confirming previous observations [13]. DP-BB rats that were fed the HC diet showed an increased TEER as compared with DP-BB rats on the standard diet (Fig. 2), confirming and extending the LA/MA data as shown in Fig. 1.

Impaired ileal mRNA expression of tight junction proteins in DP-BB rats is restored by a HC diet Tight junctions in gut epithelia are pivotal for maintenance of intestinal barrier function [27–29]. Therefore, we investigated the effect of

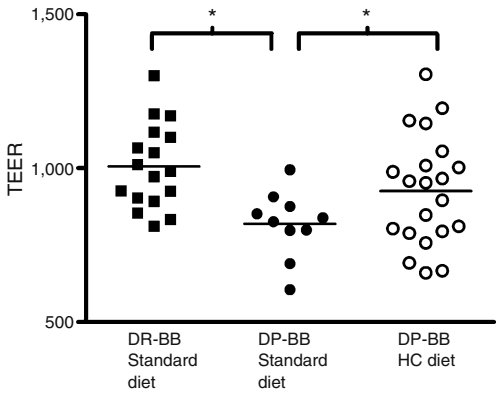

Fig. 2 DP-BB rats have reduced ileal TEER as compared with DR-BB rats or DP-BB rats fed the HC diet. Rats were killed at 65 days of age and their ileal TEER measured as described. DR-BB rats fed standard rodent diet, $n=17$; DP-BB rats on the standard diet, $n=10$; DP-BB rats on the HC diet, $n=21$. The data are presented as a scatter dot plot with the mean indicated by a horizontal line. *$p<0.05$

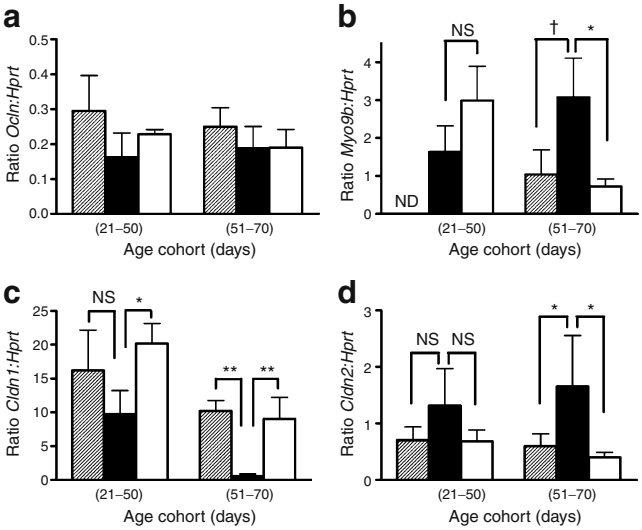

Fig. 3 Ileal mRNA expression of *Ocln* (**a**), *Myo9b* (**b**), *Cldn1* (**c**) and *Cldn2* (**d**) in DP-BB rats fed the standard diet (black bars), DP-BB rats on HC diet (white bars) and DR-BB rats fed the standard diet (hatched bars). **a** No difference in ileal *Ocln* mRNA expression between DP-BB rats fed the standard diet and those on the HC diet. **b** Increased ileal *Myo9b* mRNA expression in 51- to 70-day-old DP-BB rats fed the standard diet, as compared with other groups. **c** Reduced ileal *Cldn1* mRNA expression in DP-BB rats fed the standard diet as compared with DP-BB rats on the HC diet and DR-BB rats on the standard diet. **d** At 51 to 70 days of age DP-BB rats had increased ileal *Cldn2* mRNA expression compared with DR-BB rats. The data are presented as the mean±SEM. Group sizes: DP-BB rats 21–50 days of age (n=8), 51–70 days of age (n=8); DR-BB rats 21–50 days of age (n=5), 51–70 days of age (n=8). *p<0.05; **p<0.01; †p<0.1. ND, not determined

the HC diet on mRNA expression of *Myo9b*, *Cldn1*, *Cldn2* and *Ocln*, which encode proteins necessary for tight junction functionality.

No differences were observed regarding ileal *Ocln* mRNA expression (Fig. 3a). *Myob* expression in the ileum of DP-BB rats was increased as compared with that in DR-BB rats (Fig. 3b). Strikingly, feeding the HC diet to DP-BB rats led to a significant decrease in *Myo9b* expression in the ileum of DP-BB rats. Interestingly, the longer the DP-BB rats were fed the HC diet, the lower the expression of *Myo9b* was.

As shown in Fig. 3c, *Cldn1* expression in the ileum of DP-BB rats declined over time and at >50 days of age was lower than in DR-BB rats. Feeding the HC diet to DP-BB rats resulted in an increase of *Cldn1* expression in the ileum of the DP-BB rats. Above 50 days of age, *Cldn2* expression in the ileum of DP-BB rats was increased as compared with that in DR-BB rats (Fig. 3d). Feeding DP-BB rats the HC diet reduced the expression of *Cldn2* to levels observed in DR-BB rats on the standard diet (Fig. 3d).

Increased ileal mRNA expression of Tgf-ß and Il-10 in DP-BB rats fed a HC diet The expression of *Tgf-ß* and *Il-10* in the ileum of HC-fed DP-BB rats was increased as compared with that in DP-BB rats on the standard diet (Fig. 4). Expression in the ileum of HC-fed rats was comparable to expression levels found in the DR-BB rats. No differences were observed regarding the expression of *Ifn-γ*, *Tnf-α* and forkhead box P3 (data not shown).

Expression of ileal Cldn1 and Tgf-ß mRNA in diabetic and non-diabetic DP-BB rats The diabetic rats were killed at diabetes onset at the age of 70 to 100 days. The non-diabetic rats were killed at 140 days of age. Of the rats fed a HC diet, ten diabetic and five non-diabetic rats were analysed. Of the rats on a standard diet, ten diabetic rats and one non-diabetic rat were analysed. Only the expression of *Cldn1* and *Tgf-ß* was analysed, because the difference in expression of these variables was the most pronounced around 60 days of age.

In both the control rat groups and the HC diet-fed DP-BB rats, the diabetic and non-diabetic rats displayed lower expression of *Cldn1* and *Tgf-ß* than rats younger than 50 days of age. No differences were observed between diabetic and non-diabetic rats (ESM Fig. 1).

Reduced serum zonulin levels in DP-BB rats fed a HC diet Serum zonulin levels were measured in serum samples, which had been cross-sectionally obtained in previous experiments, of prediabetic DP-BB rats fed the standard diet or the HC diet, and killed between 50 and 70 days of age. As shown in Fig. 5, DP-BB rats receiving the HC diet had reduced serum zonulin levels as compared with rats on the standard diet. Because zonulin levels strongly correlate with intestinal permeability [12, 14, 15], this result indicates that the HC diet improves intestinal barrier function in DP-BB rats via downregulation of zonulin.

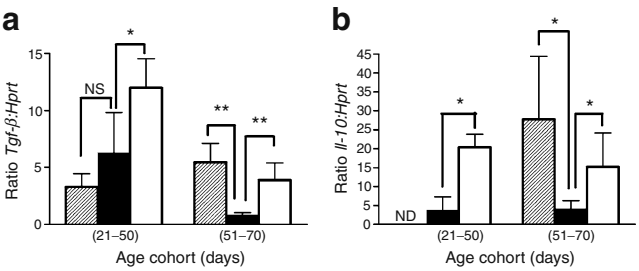

Fig. 4 **a** Increased ileal *Tgf-ß* mRNA expression in DP-BB rats on the HC diet (white bars) as compared with DP-BB rats on the standard diet (black bars). At 51 to 70 days of age DP-BB rats on the standard diet had reduced ileal *Tgf-ß* mRNA expression as compared with DR-BB rats on the standard diet (hatched bars). **b** Between 21 and 50 days of age, DP-BB rats fed the HC diet had an increased ileal *Il-10* mRNA expression as compared with age-matched DP-BB rats on the standard diet. The data are presented as the mean±SEM. Group sizes: DP-BB rats 21–50 days of age (n=8), 51–70 days of age (n=8); DR-BB rats 21–50 days of age (n=8), 51–70 days of age (n=8). *p<0.05; **p<0.01. ND, not determined

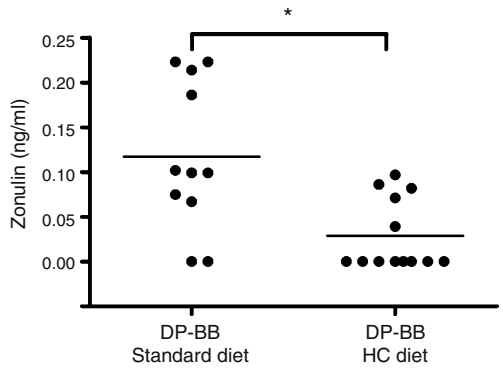

Fig. 5 DP-BB rats fed the HC diet have reduced serum zonulin levels. Zonulin levels were measured by ELISA in serum of prediabetic DP-BB rats between 50 and 70 days of age. DP-BB rats on the standard diet, $n=11$; DP-BB rats on the HC diet, $n=13$. The data are presented as scatter dot plot with the mean indicated by a horizontal line. *$p<0.05$

Discussion

Here we provide evidence that a HC diet restores the impaired intestinal barrier function in prediabetic DP-BB rats as reflected by increased ileal TEER, reduced serum zonulin levels and a reduced urinary lactulose:mannitol ratio. Interestingly, we observed an association between prediabetic intestinal permeability and diabetes outcome. Rats with a low prediabetic intestinal permeability developed autoimmune diabetes later or were protected against the disease.

Tight junctions in gut epithelia are pivotal for maintaining intestinal barrier function. Myosin IXb is an adaptor protein involved in linking the cytoskeleton with tight junctions, whereas claudins and occludin are involved in the architecture of tight junctions itself [27–29]. High levels of myosin IXb and claudin-2, and low levels of claudin-1 and occludin will result in the opening of tight junctions and a subsequent increase in intestinal permeability [27–29]. Therefore, our results for tight junction proteins may explain why, at 65 days of age, the intestinal barrier function of DP-BB rats on the standard diet is reduced compared with that of DR-BB rats. Interestingly, Watts et al. [14] demonstrated that intestinal permeability of DP-BB rats increases from the age of 50 days, which correlates with the mRNA expression pattern of tight junction proteins shown in the current report.

Besides tight junctions, mucins are also important for maintaining intestinal barrier function. It was shown by Courtois et al. that a HC diet increased intestinal mucin levels and subsequently improved intestinal barrier function in BB rats as demonstrated by reduced uptake of FITC-labelled dextran across the gut epithelium [7]. These results support those presented in this paper.

Here we show that in DP-BB rats, as opposed to DR-BB rats, the expression of *Cldn1* strongly declined over time, leading to very low levels after the age of 50 days. The mRNA expression levels of *Myo9b*, *Ocln* and *Cldn2* showed no significant changes over time, but *Myo9b* and *Cldn2* mRNA expression levels were increased as compared with DR-BB rats.

In DR-BB and DP-BB rats of 34 and 41 days of age, Neu et al. found reduced ileal claudin-1 protein levels as compared with Wistar rats, but observed no differences between DP-BB and DR-BB rats [20]. Interestingly, like Neu et al., we also found no difference in *Cldn1* mRNA expression between DP-BB and DR-BB rats below the age of 50 days. However, we did observe a difference between these two rat groups above the age of 50 days, a difference caused by the decline of *Cldn1* mRNA expression in DP-BB rats.

Interestingly, in DP-BB rats, the HC diet seemed to normalise tight junction mRNA expression levels towards levels observed in DR-BB rats. Therefore, the observed patterns of decreased *Myo9b* and *Cldn2* expression and increased *Cldn1* expression in DP-BB rats on a HC diet fits well with the hypothesis that the HC diet has a beneficial effect on gut permeability through modification of tight junction functionality.

A proinflammatory intestinal cytokine environment affects tight junction functionality and subsequently increases intestinal permeability [12, 29]. Here, the HC diet increased the anti-inflammatory cytokines IL-10 and TGF-ß, whereas TNF-α and IFN-γ levels were not affected (data not shown). The HC diet therefore creates an anti-inflammatory cytokine milieu in the small intestine, which might lead to improved intestinal barrier function.

In rats fed the standard diet and in DP-BB rats on a HC diet, the animals that did not develop diabetes had no higher ileal *Cldn1* and *Tgf-ß* expression than the diabetic rats. This result was not expected, but might be explained by ageing. As shown in Figs 3 and 4, and in ESM Fig. 1, *Cldn1* and *Tgf-ß* expression declined with age in the DP-BB rats, but not in the DR-BB rats. The non-diabetic DP-BB rats were on average 50 days older than the diabetic rats. Therefore, the comparable mRNA expression levels of *Cldn1* and *Tgf-ß* in diabetic and non-diabetic rats might have been due to ageing. Unfortunately, we did not kill non-diabetic DP-BB rats between 70 and 100 days of age.

Previous research by our group and others has shown that the HC diet affects autoimmune diabetes pathogenesis mainly between 30 and 70 days of age [1, 3]. DP-BB rats that were fed a cereal-based diet until 60 to 70 days and then switched to the HC diet were not protected against autoimmune diabetes development, suggesting a crucial window [1, 3]. Therefore, prevention of exposure to diabetogenic triggers in this window can affect diabetes outcome. The results presented here show that the decline of intestinal barrier function with age in DP-BB rats is delayed, but not completely prevented by the HC diet. However, this delay

might be enough to prevent exposure to diabetogenic triggers in the diabetes-susceptible window and subsequently prevent or delay autoimmune diabetes onset.

Food components and intestinal bacteria can affect the integrity of the intestinal barrier [8, 12, 22–24]. Recently, Mojibian et al. demonstrated that ~50% of established type 1 diabetes patients have T cell responses against wheat polypeptides [30]. These results indicate that wheat might be a major dietary antigen capable of inducing type 1 diabetes. An important question raised by the Mojibian study is why in ~50% of type 1 diabetes patients a wheat polypeptide-specific response is found. Interestingly, studies by our group and others have shown that also ~50% of type 1 diabetes patients have intestinal barrier defects [13, 15]. Although Mojibian et al. did not investigate whether the wheat-specific T cell responses correlated with impaired intestinal barrier function, it is reasonable to hypothesise that impaired intestinal barrier function leads to an increased passage of intestinal diabetogenic antigens (e.g. wheat peptides, bacterial agents) that induce the autoimmune cascade typical of type 1 diabetes [11, 12, 31]. To prove this hypothesis, further investigation of the relationship between intestinal barrier function and immune responses against intestinal antigens and beta cells in type 1 diabetes patients will be required.

In summary, the results presented here show that in DP-BB rats the level of prediabetic gut permeability is associated with autoimmune diabetes development later in life and that improvement of this intestinal barrier function might contribute to the prevention of autoimmune diabetes by dietary intervention with the HC diet. Changes to the local intestinal cytokine profile, direct effects on tight junctions and reduced zonulin production might be important mechanisms for this effect. Taken together, these results suggest that a HC-based diet probably prevents diabetes development in the DP-BB rat by: (1) improving intestinal barrier function thereby contributing to no or less induction of the autoimmune cascade responsible for autoimmune diabetes development [1, 7, 15]; (2) skewing the autoreactive T cells to a less pathogenic phenotype [1, 3, 6]; and (3) the induction of islet neogenesis [1].

Modification of intestinal permeability either directly by tight junction modulators or indirectly via environmental factors like bioactive peptides, prebiotics or probiotics [8] could be a promising approach to the development of a whole new field of therapeutic strategies to prevent or treat autoimmune diabetes.

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