

## ● Final Progress Report

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**Proposal No.** IBD-0076

**Applicant Institution:** University Hospital of Muenster (Germany)

**Project Title:** The role of the receptor for advanced glycation end products (RAGE) and its ligand S100A12 (EN-RAGE) in chronic inflammatory bowel disease

**Award Period:** November 1, 2003 - June 30, 2006

### i. Summary of Project Aims

The overall goal of this project has been to understand the role of the neutrophil-derived calcium-binding protein S100A12 for the interaction of leukocytes with endothelial cells during inflammation in IBD. Based on our previous findings we hypothesized that S100A12 is a significant modulator of the aberrant inflammatory response that leads to chronic inflammation and tissue damage in IBD.

The project was divided into three major parts. One goal of this application was to understand the molecular events that involve S100A12 and its receptor RAGE on endothelial cells. We also investigated the relevance of RAGE polymorphisms for the susceptibility to develop IBD or for the risk of complications during the course of the disease. Another goal was to evaluate the usefulness of S100A12 as a serum marker for the inflammatory disease activity and prognosis related to IBD. These goals were achieved in experiments following 3 specific aims:

***Characterization of the RAGE-mediated effects of S100A12 on endothelial cells and leukocytes.*** By analyzing the molecular basis of the S100A12 action and by evaluating changes in gene expression of endothelial cells and leukocytes, we aimed to identify whether S100A12 is a critical player in the development of IBD and a target for therapies.

***Determination of the influence of RAGE polymorphism on the susceptibility to develop IBD and on the course of the disease.*** We investigated whether the RAGE receptor itself is a key molecule which may function aberrantly in patients with IBD?

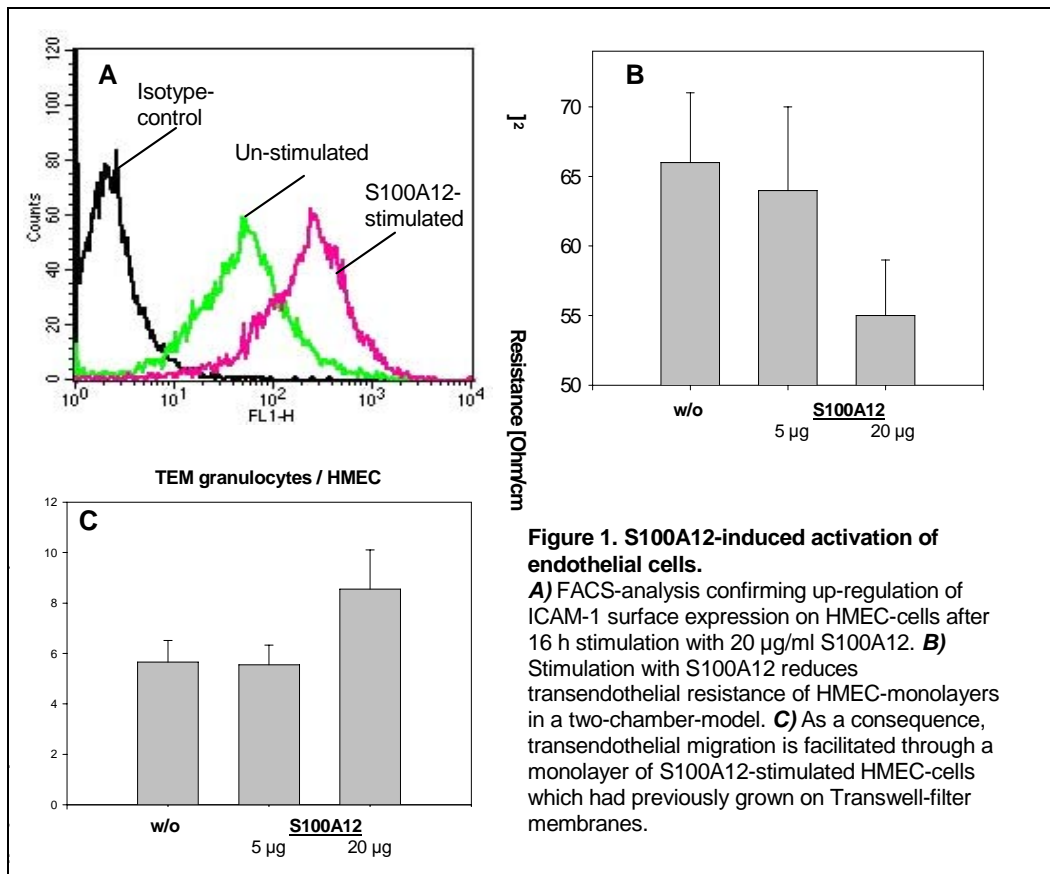
***Evaluation of the prognostic value of S100A12 serum analyses for the further course of IBD, especially the occurrence of relapses or complications.*** We analyzed whether the S100A12 serum test provides a valuable diagnostic test to determine disease activity in patients with IBD.

### ii. Accomplishments towards Meeting the Aims

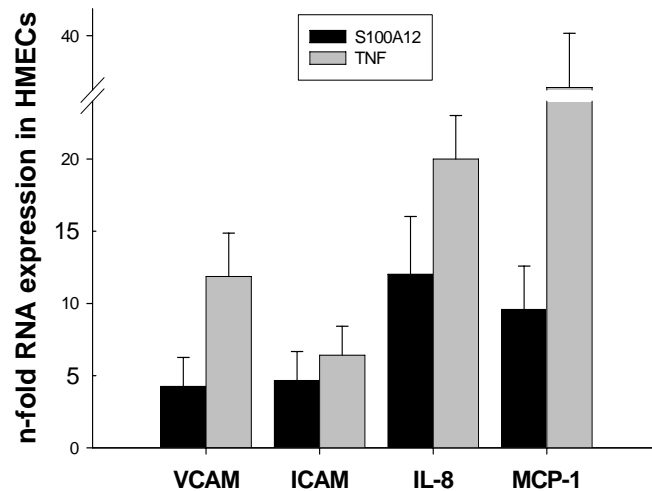
We have made strong efforts in our studies. These efforts resulted in achievements essential for the three steps outlined in the research plan.

***Characterization of the RAGE-mediated effects of S100A12 on microvascular endothelial cells and leukocytes.*** For the in vitro-studies, purification and thorough characterization of sufficient amounts of the S100A12 protein was a prerequisite. We have purified native human S100A12 and also recombinant protein, which exerts potent biological activity.

The influence of S100A12 on transendothelial migration properties of inflammatory cells was analyzed using human microvascular endothelial cells (HMEC). We have performed experiments confirming changes in response to S100A12 stimulation, especially adhesion molecule upregulation and changes in transendothelial integrity resulting in enhanced transendothelial migration of leukocytes. To analyze if changes in transendothelial migration depend on reduced tightness of the endothelial barrier function, we analyzed the electrical resistance of the endothelial monolayer (Fig. 1).



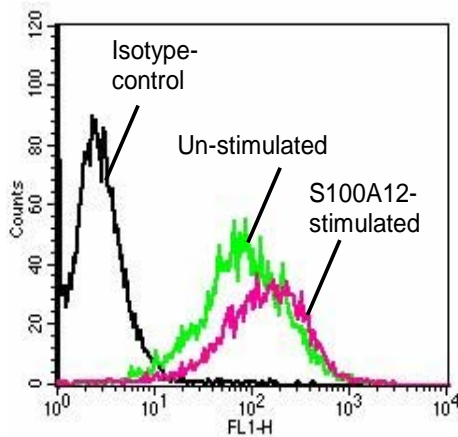
The production of chemoattractants by endothelial cells is a mean by which activated endothelium recruits more leukocytes to sites of inflammation. We analyzed the gene expression adhesion molecules, IL-8, and other products like MCP-1 by HMEC monolayer in response to S100A12 and TNF (Fig. 2).



**Figure 2. Expression of adhesion molecules and chemokines.**

Real-Time PCR for mRNA expression of ICAM-1, IL-8, and MCP-1 in S100A12- and TNF $\alpha$ - stimulated HMEC-cells (relative expression compared to un-stimulated controls, normalized to GAPDH). S100A12-stimulation leads to a significant up-regulation of chemokine-production by endothelial cells.

In addition, monocytes stimulated with S100A12 show increased secretion of IL-8 (not shown). We have also performed analyses on primary endothelial cells derived from human intestine (Fig. 3).



**Figure 3. S100A12-induced activation of primary intestinal endothelial cells.**

FACS-analysis confirming up-regulation of ICAM-1 surface expression on primary isolated human intestinal microvascular (HIMEC)-cells after 16 h stimulation with 20  $\mu$ g/ml S100A12.

**Determination of the influence of the G82S RAGE polymorphism on the susceptibility to develop IBD and on the course of the disease.** The association of Single Nucleotide Polymorphisms (SNPs) of the RAGE gene with IBD has been studied.

We have analyzed a total of 635 DNA samples from patients with IBD and 538 controls. We have obtained results which make it unlikely that an association with of the G82S RAGE SNP with IBD exists (Tab. 1). We were looking for additional SNPs within the RAGE Gene (-374T/A, Promotor). Obviously, the -374T/A RAGE promotor SNP has a protective effect on the susceptibility for Crohn's disease (CD). Data were correlated with subgroups of patients with complications and other characteristics of

patients. At the moment, we aim in confirming our results in a second cohort (in cooperation with Uli Broeckel and Subra Kugathasan at the Medical College of Wisconsin).

**Table 1.** Analysis of polymorphism in the RAGE gene

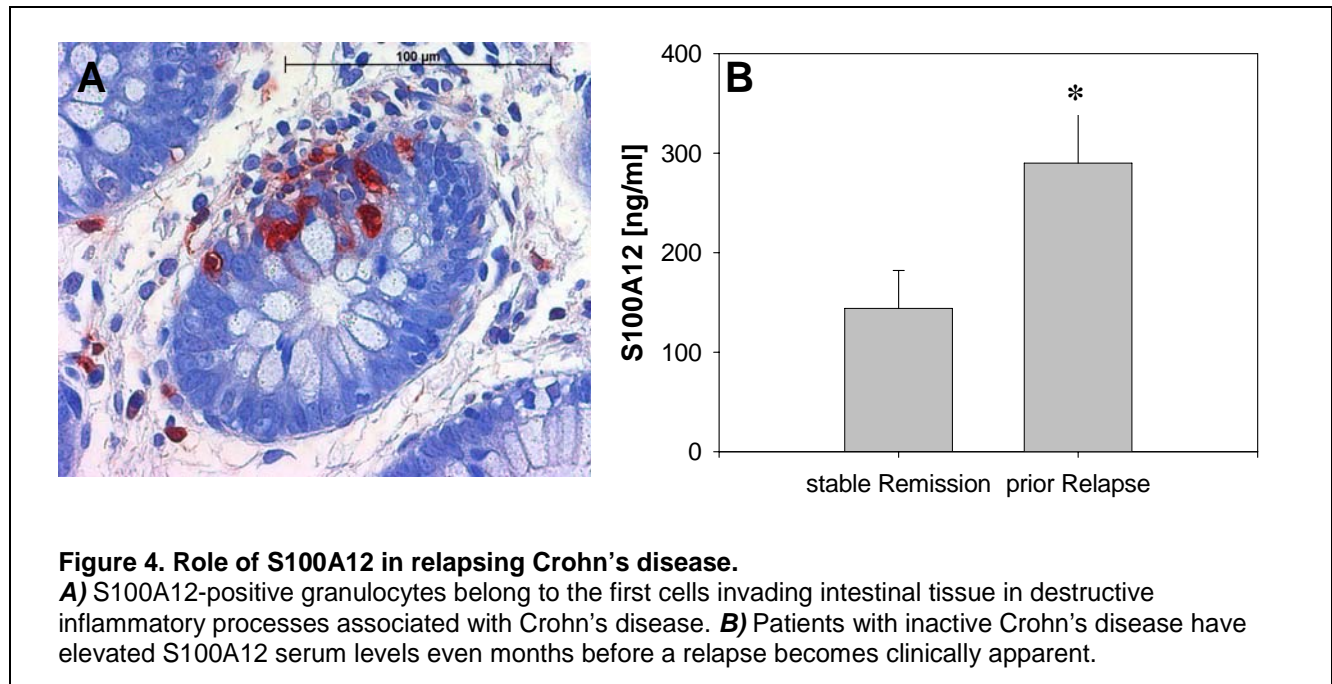
	<i>odds ratio</i>	<i>95% CI</i>	<i>p</i>
<b>G82S CD</b>	<b>1.22</b>	<b>0.69-2.15</b>	<b>n.s.</b>
<b>UC</b>	<b>0.68</b>	<b>0.30-1.52</b>	<b>n.s.</b>
<b>-374T/A CD</b>	<b>0.71</b>	<b>0.53-0.93</b>	<b>0.016</b>
<b>UC</b>	<b>0.99</b>	<b>0.71-1.38</b>	<b>n.s.</b>

**Evaluation of the prognostic value of S100A12 serum analyses for the further course of IBD, especially the occurrence of relapses or complications.** In previous studies we found that the cohort of IBD patients in remission as a whole had higher mean S100A12 serum levels than healthy controls. Hence, we speculated that this group comprises a heterogeneous group with some patients who have residual inflammation not leading to clinically apparent signs of disease activity but nevertheless implying a risk for developing a relapse.

S100A12 may be a predictive marker for the risk of relapse or complications during the further course of the disease. Taking in account the reported usefulness of “fecal calprotectin” (a heterocomplex of S100A8 and S100A9) as a biomarker, it is intriguing that neutrophil-specific S100A12 could be a feasible non-invasive diagnostic test for adults and especially for children suffering from IBD. Based on these observations, we investigated the diagnostic accuracy of fecal S100A12 in the determination of intestinal inflammation. Currently, the variation of fecal “calprotectin” assays still impedes the routine use of this marker as a sole parameter in clinical practice. The observed variation may be due to the broad expression pattern of S100A8/S100A9 which is also inducible in epithelial cells. In this context the elevation in lactose intolerance is notable. Furthermore, it is not known whether epithelial cells constitutively express these proteins under certain conditions. Our preliminary studies suggest that fecal S100A12, which is more restricted to neutrophils, is a better marker for making the diagnosis and for monitoring disease activity than fecal S100A8/S100A9 (“calprotectin”).

In our study we were including 83 adults with suspected IBD who underwent endoscopy (see supplement 2). Stool was obtained from these patients at initial presentation (i.e. before any therapies were initiated). During endoscopy, biopsies were obtained for standard histology and also for immunohistochemistry using specific antibodies directed against S100A12, S100A8/S100A9, neutrophils markers (CD15, elastase), and monocyte marker CD68. Endoscopic details and scores, clinical features, and routine laboratory data (including blood counts, CRP, ESR) were recorded. We could confirm the excellent correlation of S100A12 serum concentrations to disease activity of IBD. In addition, this marker can differentiate between IBD and IBS. As a completely novel finding we could for the first time show that S100A12 was detectable in stool of patients with active IBD, while it was significantly lower in individuals with IBS. In IBD, fecal S100A12 levels correlate well to disease activity scores. Fecal S100A8/S100A9 was also elevated in IBD. In contrast to S100A12, fecal S100A8/S100A9 was not able to differentiate between IBD and IBS (Supplement 2). Preliminary results have also been presented at the last Broad Investigator meeting. More details on our results can be

found in supplement 2. Fecal S100A12 has been recalculated to mg/kg taking in account the stool specimen preparation procedure, analogue to fecal calprotectin.



### iii. List of Significant Results

1. The neutrophil-derived protein S100A12 is a pro-inflammatory mediator. It activates cells which are crucial for the pathogenesis of IBD. Activation of leukocytes and endothelium may facilitate further recruitment of immune cells into inflamed intestinal tissue. Our results are part of a manuscript submitted to the *Journal of Pathology* (**supplement 1**).
2. Certain genetic variations of the S100A12-receptor RAGE may alter the susceptibility for IBD. In particular, the -374T/A RAGE promoter polymorphism is negatively linked to CD. At the moment, we aim in confirming these results in a second cohort prior to potential publication.
3. S100A12 levels in serum and stool correlate strongly to IBD disease activity. Analyzing S100A12 concentrations may serve as a useful diagnostic tool in the monitoring of inflammation. Our results are part of a manuscript submitted to *Gastroenterology* (**supplement 2**).

We feel that the results of this study are very significant, and they offer a possible avenue for monitoring disease activity or treatment of IBD. Understanding the mechanism by which S100A12 aggravates inflammation specifically in IBD is within reach.

The combination of basic science using the in vitro models together with clinical science analyzing patient samples has increased the understanding of how the pro-inflammatory S100A12 may influence the establishment and perpetuation of IBD symptoms.

Moreover, S100A12 is detectable in stool during intestinal inflammation, and its fecal concentration strongly correlates to disease activity. This protein can significantly improve our arsenal of noninvasive biomarkers of intestinal inflammation.

## vi. Lay Summary

The underlying factors causing chronic inflammatory bowel disease (IBD) are still poorly understood. However, we do know that white blood cells invade bowel tissue and contribute to an excessive inflammation during IBD. In order to leave the blood stream and invade the bowel tissue, inflammatory cells attach to interior cells of intestinal blood vessels and finally pass this cell lining that normally functions as a barrier between tissue and the blood stream. One factor contributing to this interaction between white blood cells and the blood vessel wall is a protein called S100A12 (or calgranulin C; EN-RAGE) that is secreted by inflammatory cells.

The aim of our studies was to identify serum markers that help assessing the disease activity of chronic IBD. Correct assessment of the activity of inflammation in individual patients is important to guide therapy which has to balance the risks of under- and over-treatment. We have identified that S100A12 - produced and released by the most abundant human white blood cells called neutrophils – is a potential marker for the disease activity of IBD. We have demonstrated that S100A12 is clearly elevated in the blood of patients with active IBD. In addition, we found that S100A12 decreased to almost normal in times when the disease was completely inactive. Furthermore, S100A12 is detectable in stool during intestinal inflammation. This protein can significantly improve our arsenal of noninvasive biomarkers of intestinal inflammation.

The primary clinical benefit of our studies will thus be the generation of more sophisticated diagnostic markers for IBD. The correct assessment of the disease activity will help to guide treatment. In the future, S100A12 may also serve as a new target for therapeutic interventions. This protein initiates inflammation via cell surface receptors found in small blood vessels. We will find that the effects of S100A12 directly mediate inflammation by changing the function of small blood vessels. This leads to new insights into the underlying mechanisms causing IBD. The receptor of S100A12 (called RAGE) is important for the response of immune cells to stimulation by S100A12, and RAGE exists in certain genetically determined variants which influence the susceptibility of individuals for developing Crohn's disease. The binding of S100A12 to its receptor RAGE is a crucial step, and this interaction might be blocked by future drugs that can be used to treat patients with chronic IBD.