POTENTIAL DIAGNOSTIC BIOMARKERS OF ULCERATIVE COLITIS-ASSOCIATED COLORECTAL DYSPLASIA

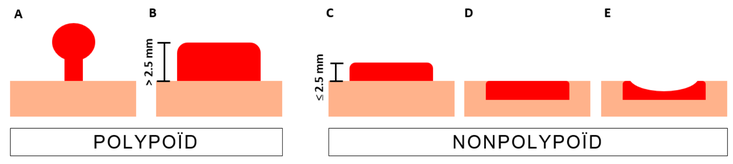
MERLI A-M[1], MASSOT C[2], BLETARD N[3], QUESADA CALVO F[1], BAIWIR D[4], MAZZUCCHELLI G[5], SMARGIASSO N[5], SERVAIS L[6], OURY C[6], DE PAUW-GILLET M-C[7], DE PAUW E[5], COIMBRA MARQUES C.[8] COLARD A[9], VIJVERMAN A[10], DELVENNE P[3], MEUWIS M-A[1] AND LOUIS E[1]

1. GIGA-Research, Translational Gastroenterology Uliège, Liège (Belgium); 2. CHU de Liège, Hepato-Gastroenterology and Digestive Oncology, Liège (Belgium); 3. CHU de Liège, Pathological anatomy and cytology, Liège (Belgium); 4. ULiège, GIGA Proteomic Facility, Liège (Belgium); 5. ULiège, Laboratory of Mass Spectrometry, Chemistry, Liège (Belgium); 6. GIGA-Research, Laboratory of Thrombosis and Hemostasis, ULiège, Liège (Belgium); 7. ULiège, Liège, Mammalian Cell Culture Laboratory (Belgium); 8. CHU de Liège, Abdominal Surgery Department, Liège (Belgium); 9. CHC de Liège, Gastroenterology, Liège (Belgium); 10. CHR Citadelle de Liège, Gastroenterology and Digestive Oncology, Liège, Belgium

Contact : am.merli@uliege.be

CLINICAL CONTEXT

Patients suffering from inflammatory bowel diseases (IBD), such as Ulcerative Colitis (UC) and Crohn's disease (CD), can develop dysplasia which is a pre-cancerous lesion of the colon or the rectum. Dysplasia can itself progress and transform into neoplasia and lead to colorectal cancer. In IBD, dysplasia can develop on areas that are or have been affected by chronic inflammation; in this setting, the dysplasia is termed Dysplasia Associated to Inflammation (DAI) (**figure 1**). Dysplasia may also develop independently of chronic inflammation and is termed Sporadic Dysplasia (DSp). Anatomopathological diagnosis of DAI in IBD patients remains difficult, especially when tissue inflammation is present, as regenerative remodeling in the mucosae is observed and impairs dysplasia confirmation.



▲ **Figure 1: SCENIC international consensus for classification of dysplasia in the context of IBD** (Laine L. *et al*, Gastroenterology 2015). (A) polypoid pedunculated lesion, (B) polypoid sessile lesion, (C) nonpolypoid superficial elevated lesion, (D) nonpolypoid flat lesion and (E) nonpolypoid depressed lesion.

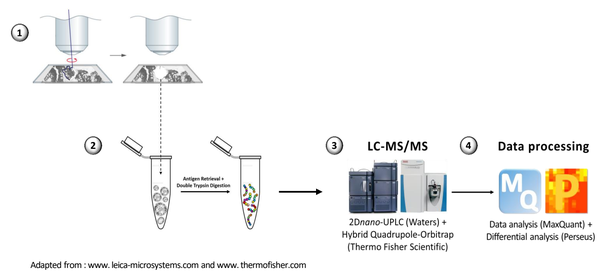
PILOT STUDY IN UC

Research strategy: we performed a proteomic pilot study on 5 UC cases, including paired samples. Some results were confirmed by immunohistochemistry (IHC) on a higher number of patients. Finally, mouse model of colitis-associated cancer (CAC) was used to investigate one potential biomarker highlighted by proteomics to evaluate its distribution in different lesion types covering inflammation, DAI and adenocarcinoma.

We performed a pilot experiment on 15 Formalin-Fixed, Paraffin-Embedded (FFPE) samples isolated from 5 cases of UC patients with DAI (**table 1**). Samples were treated following the workflow illustrated in **figure 2**. We compared the proteomes of the dysplastic (DAI), the inflammatory (I) and the normal (NL) tissues of each patient.

Clinical data - proteomic pilot experiment (n=5) and human immunohistochemistry (n=11)					
Men, n (%)	4 (80)	9 (81.82)	**Morphology of dysplasia (SCENIC Consensus)**		
Age, year, median (P25-P75)	62 (57-64)	56 (45.5-66)	***Polypoid***		
Smokers, n (%)	0 (0)	0 (0)	*Pedunculated*	5 (100)	9 (81.81)
Inflammatory Bowel Disease			*sessile*	0 (0)	1 (9.09)
Ulcerative Colitis, n (%)	5 (100)	11 (100)	***Nonpolypoid***		
DAI, n (%)	5 (100)	11 (100)	*superficial elevated*	0 (0)	0 (0)
DSp, n (%)	0 (0)	0 (0)	*flat*	0 (0)	1 (9.09)
Crohn's disease, n (%)	0 (0)	0 (0)	*depressed*	0 (0)	0 (0)
Localisation of dysplasia			**Size of the lesions**, mm, median (P25-P75)	5 (5-8)	7 (4.5-18)
Colon	5 (100)		**Medications**		
Ceacum, n(%)	2 (40)	4 (36.36)	***Anti-inflammatory drugs***		
Ascending colon, n (%)	2 (40)	2 (18.18)	*Mesalamine, n (%)*	3 (60)	0 ('0)
Transverse colon, n (%)	0 (0)	0 (0)	*Mesalazine, n (%)*	0 (0)	5 (45.45)
Descending colon, n (%)	0 (0)	2 (18.18)	***Immunomodulators***		
Sigmoid colon, n (%)	1 (20)	3 (27.27)	*Azathioprine, n (%)*	1 (20)	1 (9.09)
Rectum, n (%)	0 (0)	0 (0)	*Methotrexate, n (%)*	1 (20)	2 (18.18)
Grade of dysplasia			*Anti-TNF, n (%)*	1 (20)	2 (18.18)
Low grade, n (%)	5 (100)	9 (81.82)			
High grade, n (%)	0 (0)	2 (18.18)			

▲ **Table 1: A clinical data of the patients included in the proteomic pilot experiment and in human immunohistochemistry.**



Adapted from : www. leica-microsystems.com and www. thermofisher.com

▲ **Figure 2: proteomic experiment workflow.** (1) Zones of interest were collected by Laser-Capture Microdissection. (2) Samples were submitted to antigen retrieval for a downstream two-step trypsin digestion. (Longuespée R. *et al.*, Methods 2016) (3) The peptide mixtures were analysed after separation by Ultra Performance Liquid Chromatography in two dimensions, by an Hybrid Quadrupole-Orbitrap mass spectrometer. (4) Raw data were treated using the MaxQuant software (Cox J., Mann M., Nat Biothechnol, 2008) and the differential analysis was performed with Perseus software (Tyanova S. *et al*, Nat Methods, 2016).

RESULTS

1 Proteomics:

Out of 985 quantified proteins, 7 were significantly more abundant in dysplastic tissues (**table 2**).

DAI only	DAI>I
Solute carrier family 12 member 2 (SLC12A2)	60 kDa heat shock protein, mitochondrial (hsp60) p value = 0.002 ratio D/I = 1.695
Hepatoma-derived growth factor (HDGF)	
Cytochrome c	
Proteasome activator complex subunit 1	
MICOS complex subunit MIC60	
3-hydroxyisobutyryl-CoA, mitochondrial (HIBYL-CoA)	

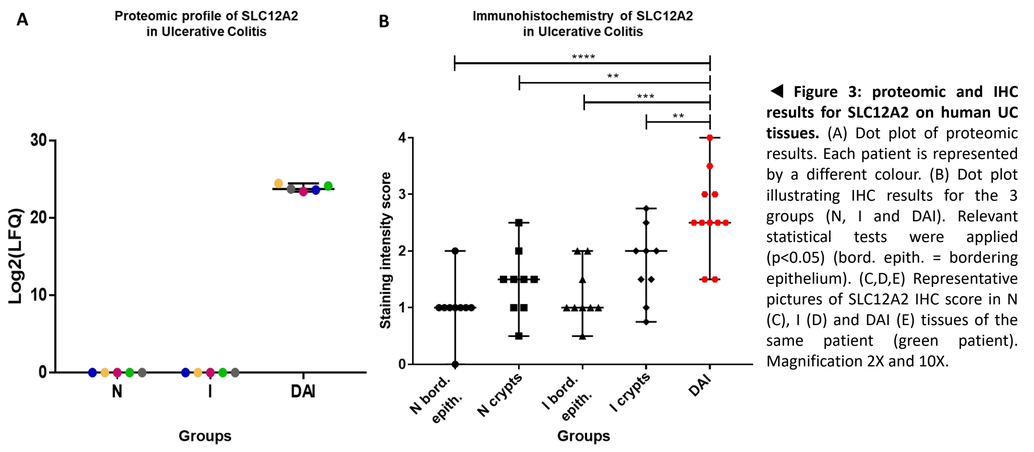
◀ **Table 2: proteins found significant in Dysplastic *vs* Inflammatory tissues.** Proteins quantified only in the dysplastic group ("DAI only") and protein quantified in both groups significantly more abundant in the Dysplastic one ("DAI>I"), using paired t-test ($p<0.05$).

SLC12A2 in literature:

The protein is also called Na-K-2Cl co-transporter 1 (NKCC1) due to its function as transporter of 1 Na^+, 1 K^+ and 2 Cl^- into the cell. It is involved in ionic balance and cell volume regulation [1] and is influenced by physical, hormonal, signaling and cytokines factors. It is implicated in T cells migration via WNK1-OXSR1/STK39-SLC12A2. [2] ***In cancer*** *: it is 3 times more abundant in colorectal cancer than in normal tissue. [3] In lung adenocarcinoma, its increase is correlated to more advance and aggressive lesions. [4] In gastric adenocarcinoma, its is associated with poor tumor differentiation. [5] In human glioma cells, its inhibition induces reduction of migration and invasion. [6]*

2 Immunohistochemistry for SLC12A2 distribution in UC patients samples and comparison to proteomic results:

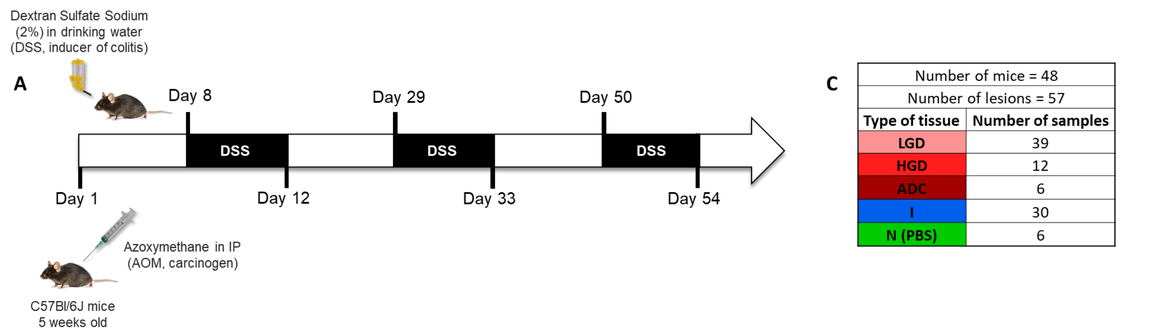
IHC evaluation included 11 UC-DAI paired samples: I (*n=9*) and/or NL (*n=9*) tissues. Staining intensity scale used ranges from 0 to 4 (0 = none, 1 = low, 2 = medium, 3 = high and 4 = very high). SLC12A2 was more intense in dysplasia in both IHC and proteomics (**figure 3AB**). Results show that SLC12A2 staining score is more intense in dysplastic than in inflammatory (crypts: p=0.002, bordering epithelium: p=0.0001) and normal tissues (crypts: p=0.001, bordering epithelium: p<0.0001) (**figure 3B**). SLC12A2 staining in the DAI tissue allows clear delimitation of the dysplastic region from surrounding inflammatory tissue (see red doted line in **figure 3E**).



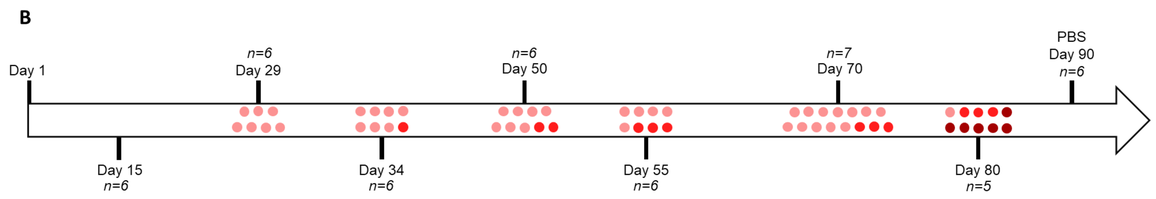
◀ **Figure 3: proteomic and IHC results for SLC12A2 on human UC tissues.** (A) Dot plot of proteomic results. Each patient is represented by a different colour. (B) Dot plot illustrating IHC results for the 3 groups (N, I and DAI). Relevant statistical tests were applied ($p<0.05$) (bord. epith. = bordering epithelium). (C,D,E) Representative pictures of SLC12A2 IHC score in N (C), I (D) and DAI (E) tissues of the same patient (green patient). Magnification 2X and 10X.

3 Characterisation of SLC12A2 in dysplasia and adenocarcinoma in the AOM/DSS mouse model:

C57Bl/6J mice were injected intraperitoneally with the carcinogen agent Azoxymethane (AOM) diluted in PBS. Chronic inflammation was induced by 3 cycles of 5 days of Dextran Sulfate Sodium (DSS) 2% in their drinking water. Mice were euthanized at different time points (see **figure 4B** for exact number of mice per time point). Colon and rectum were used as FFPE material. Classification of all tissues and lesions was established by a trained anatomopathologist specialized in gastroenterology (**figure 4**).



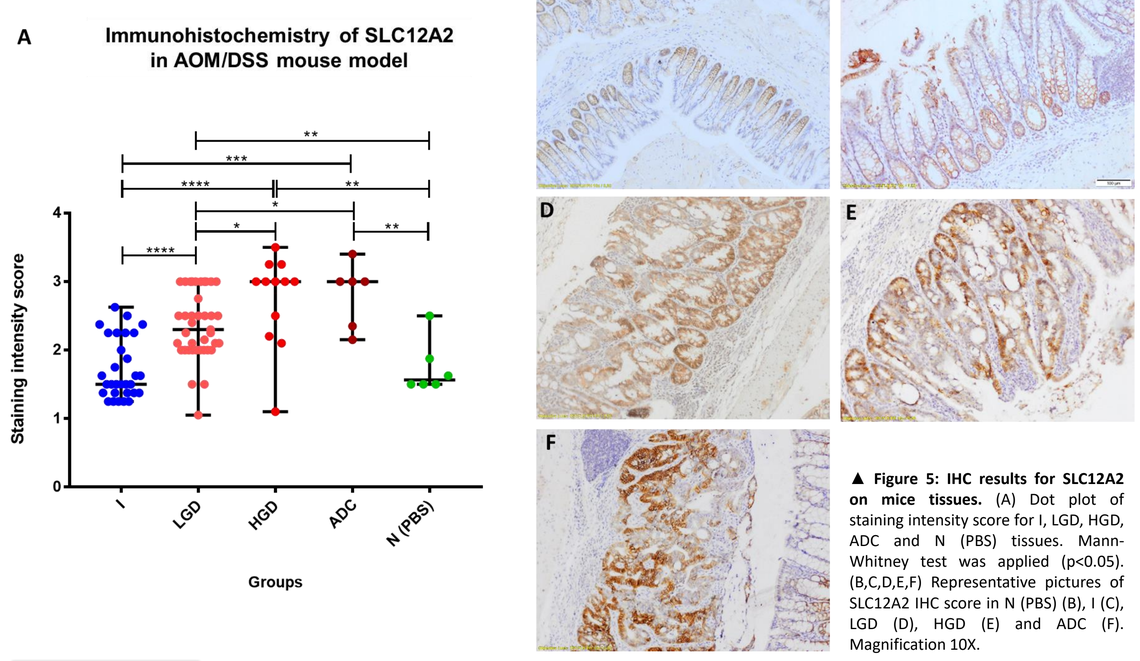
Number of mice = 48	
Number of lesions = 57	
Type of tissue	**Number of samples**
LGD	39
HGD	12
ADC	6
I	30
N (PBS)	6



▲ **Figure 4: AOM/DSS mouse model.** (A) Schematic representation of the mouse model with AOM and DSS treatment cycles. (B) Mice were euthanized at days 15, 29, 34, 50, 55, 70 and 80 (*n=6, 6, 6, 6, 6, 7, and 5* respectively). Mice (n=6) were treated with PBS and sacrificed at day 90 as controls. Each point represent one lesion (*see color code on* ***figure 4C***). (C) Number of samples obtained for each type of tissue., with N = normal, I = inflammation, LGD = low grade dysplasia, HGD = high grade dysplasia and ADC = adenocarcinoma.

4 Evaluation of SLC12A2 distribution in colonic tissues of the AOM/DSS mouse model:

IHC staining intensity scale ranges from 0 to 4 as in UC patients. Staining intensity score of SLC12A2 was significantly more important in dysplastic lesions (*n=52*) than in inflammatory (I) (*n=30*) and in normal (N (PBS)) tissues (*n=6*). IHC scores were significantly increased in advanced lesions HGD (*n=12*) and ADC (*n=6*) compared to LGD (*n=39*) (**figure 5**).



▲ **Figure 5: IHC results for SLC12A2 on mice tissues.** (A) Dot plot of staining intensity score for I, LGD, HGD, ADC and N (PBS) tissues. Mann-Whitney test was applied ($p<0.05$). (B,C,D,E,F) Representative pictures of SLC12A2 IHC score in N (PBS) (B), I (C), LGD (D), HGD (E) and ADC (F). Magnification 10X.

CONCLUSIONS

- 7 proteins were found more abundant in DAI than in I paired tissues by differential label free proteomics
- SLC12A2 was only quantified in the DAI group by proteomics
- IHC of SLC12A2 in human UC-DAI paired samples confirmed our proteomic results
- In UC, staining of SLC12A2 was significantly more intense in DAI than in I and N paired tissues
- In UC, staining of SLC12A2 allowed delimitation of DAI lesion from the surrounding I tissue
- SLC12A2 detected by IHC showed an association with dysplasia and neoplasia in CAC mouse model
- HGD and LGD tissues showed a significant higher staining of SLC12A2 than LGD lesions of the CAC mouse model

CONCLUSION

SLC12A2 could be a potential marker of DAI in UC as being able to identify dysplasia and neoplasia from surrounding tissues with inflammation. SLC12A2 requires proper validation to evaluate its power as a specific IHC marker that could be used to clarify difficult cases diagnosed as "indefinite for dysplasia".

References : [1] Markadieu N. and Delpire E., Pflugers Arch (2014). [2] Köchl R. *et al*, Nat Immunol (2016). [3] Zhang Y. et al, J Cancer Res Clin Oncol (2016). [4] Sun P.L. et al, QJM (2016). [5] Shiozaki A. et al, J Physiol Sci (2006). [6] Haas B.R. and Sontheimer H., Cancer Res (2010).

This work was made possible thanks to the financial supports of Roche and MSD. *A special thanks to L Trzpiot and N Rosière for technical assistances, to the Biothèque of ULg for the FFPE sample preparation (S Gofflot, K El Kandoussi and R Thonon), to the Imaging plateform (Sandra Ormenese and Jean-Jacques Goval) and to the Immunohistology platform (Chantal Humblet, Alice Marquet and Tiffany Di Salvo).*