

1 ***In vitro* profiling of endocrine cell death using UCHL1 and GAD65 as**  
2 **soluble biomarkers**

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## 22 Abstract

23 **BACKGROUND:** Pancreatic islet grafts are cultured *in vitro* prior to transplantation and this  
24 is associated to a variable degree of beta cell loss. Optimization of culture conditions is  
25 currently hampered by the lack of a specific and sensitive *in vitro* indicator of beta cell death.

26 **METHODS:** We developed a high-sensitivity duplex bead-based immunoassay for two  
27 protein-type biomarkers of beta cell destruction, GAD65 and UCHL1, and investigated its  
28 proficiency for *in vitro* toxicity profiling on rodent and human beta cells, as compared to a  
29 semi-automatic and manual image-based assessment of beta cell death, and *in vivo* after  
30 intraportal islet transplantation.

31 **RESULTS:** Both GAD65 and UCHL1 were discharged by necrotic and apoptotic beta cells  
32 proportionate to the number of dead beta cells as counted by microscopic methods. *In vitro*,  
33 UCHL1 was superior to GAD65, in terms of biomarker stability providing more sensitive  
34 detection of low grade beta cell death. *In vivo*, however, GAD65 was consistently detected  
35 after islet transplantation while UCHL1 remained undetectable.

36 **CONCLUSION:** The use of soluble biomarkers represents a fast, selective and sensitive  
37 method for beta cell toxicity profiling *in vitro*. UCHL1 is superior to GAD65 *in vitro* but not *in*  
38 *vivo*.

## 39 Introduction

40 Intra-portal transplantation of human islets in chronic type 1 diabetes (T1D) patients can  
41 improve metabolic control and quality of life [1]. Donor-derived grafts are cultured *in vitro*  
42 prior to transplantation under standardized culture conditions i) to allow preferential survival  
43 of functional beta cells and deplete the preparations of debris, acinar cells, leukocytes and  
44 major histocompatibility complex (MHC)-class II positive cells; ii) to determine the cellular  
45 composition of the grafts by immunocytochemistry and electron microscopy; iii) to define

46 functional properties of individual human beta cells (DNA and insulin content, rate of glucose-  
47 induced insulin release and biosynthesis) and finally iv) to be able to pool grafts from various  
48 donors in order to transplant a sufficient number of insulin secreting cells, e.g. according to  
49 our experience minimally  $2 \times 10^6$  cells per kilogram of body weight [2–4]. Long-term *in vitro*  
50 culture of islet grafts is, however, also associated to a variable degree of beta cell loss.  
51 Optimization of culture conditions to minimize beta cell loss is currently hampered by the lack  
52 of a specific and sensitive indicator of beta cell toxicity that can be used in high-throughput.  
53 Here we describe the *in vitro* performance of a newly developed duplex immunoassay for two  
54 protein-type indicators of beta cell injury. Specificity for beta cell-selective death was  
55 achieved by combining two previously described protein-type biomarkers of beta cell  
56 destruction: the classical beta cell auto-antigen 65kDa subunit of glutamate decarboxylase  
57 (GAD65), and the neuroendocrine-selective ubiquitin carboxy-terminal hydrolase 1 (UCHL1)  
58 [5, 6]. Both proteins are reported to be highly beta cell selective and absent in all exocrine  
59 cells (acinar and duct) and other endocrine (alpha) cells. High analytical sensitivity was  
60 achieved through the development of a duplex enhanced-sensitivity cytometric bead assay  
61 (CBA). Implemented on less sensitive immunoassay platforms, both biomarkers were  
62 previously successful for the detection and quantification of massive, synchronous necrotic  
63 beta cell destruction *in vitro* and *in vivo* [5, 7]. However, during late-stage islet graft rejection,  
64 during the natural course of T1D [8] and during long-term islet graft culture *in vitro*, apoptosis  
65 is likely the main form of beta cell death Here we investigated the ability of the newly  
66 developed sensitive duplex UCHL1-GAD65 CBA assay in comparison to manual or semi-  
67 automated high-content microscopy using vital dyes as gold standard, to detect and quantify  
68 - rat beta cell apoptosis induced *in vitro* by a pathophysiologically relevant cocktail of pro-  
69 inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ ) [9–11], or after induction of endoplasmic  
70 reticulum stress by thapsigargin [9, 12–15]. We found that *in vitro* profiling of UCHL1-  
71 GAD65 discharge was not only more sensitive and accurate than microscopy-based  
72 methods, but also has a broader application field e.g. the time-resolved monitoring of cell  
73 death of beta cells physiologically cultured as tight endocrine aggregates.

## 74 **Materials and methods**

### 75 **Beta cell isolation**

76 Rat beta cells were FACS-purified from islet-cell enriched suspensions [16] prepared from  
77 adult (8 or 10-week-old, male) Wistar rats (Janvier Bioservices, France). Beta cell  
78 preparations consisted of  $\geq 95\%$  endocrine cells (90% insulin+, 3% glucagon+, 1%  
79 somatostatin+ and 2% pancreatic polypeptide+ cells) and  $< 2\%$  exocrine cells. Beta cells  
80 were cultured in Ham's F10 media (Thermo Fisher Scientific Inc., USA) supplemented with 2  
81 mmol/L L-glutamine, 50 mmol/L 3-isobutyl-1-methylxanthine, 0.075 g/L penicillin (Sigma  
82 Aldrich, USA), 0.1 g/L streptomycin (Sigma Aldrich, USA), 10 mmol/L glucose, and 10 g/L  
83 charcoal-treated bovine serum albumin (factor V, radioimmunoassay grade; Sigma Aldrich,  
84 USA).

### 85 **In vitro toxicity profiling**

86 Isolated single rat beta cells (6000 cells/well in 100  $\mu$ L medium) were cultured in  
87 poly-D-lysine-coated 96-well plates (Thermo Fisher Scientific Inc., USA). After seeding, the  
88 cells were exposed (on day 0) for 3 to 6 days to the following toxins: i) thapsigargin (0.2  $\mu$ M  
89 final concentration, Sigma Aldrich, USA); ii) a combination of three cytokines: recombinant  
90 TNF- $\alpha$ , recombinant human IL-1 $\beta$  and recombinant IFN- $\gamma$  (all three used at 1ng/mL final  
91 concentration, R&D Systems, USA) and iii) hydrogen peroxide (100mM H<sub>2</sub>O<sub>2</sub> final  
92 concentration, Merck KGaA, Germany). Medium was replaced on day 3. At day 3 and day 6  
93 supernatant samples were taken for biomarker analysis with duplex cytometric bead array  
94 (CBA) and cell viability was assessed according to following methods.

### 95 **Cell viability – automatic versus manual**

96 The percentages of viable, necrotic, and apoptotic cells were assessed in the single cell  
97 preparations after a culture period of 3 to 6 days. For this purpose, the cells were exposed to  
98 the DNA binding dyes Hoechst 33342 (Ho; Sigma Aldrich, USA) and propidium iodide (PI;  
99 Sigma Aldrich, USA)[14]. Hoechst 33342 readily enters cells with intact membranes as well

100 as cells with damaged membranes whereas uptake of the highly polar dye PI is restricted to  
101 cells with loss of plasma membrane potential, staining only dead cells. After 15 min of  
102 incubation at 37°C with 5% CO<sub>2</sub>, cell viability was assessed both automatically (operator  
103 independent) and manually (operator dependent). Absolute number of cells was determined  
104 automatically by whole-well imaging using Pathway 435 (BD Biosciences, USA) (Fig 1A).  
105 Acquisition of pictures using customer defined assays is done with Attovision software (BD  
106 Biosciences, USA). After fine autofocus of Hoechst stained nuclei, a whole well montage of 8  
107 by 12 pictures is taken from the Hoechst channel and from the PI -channel. Pictures are  
108 analysed by Attovision software (BD Biosciences, USA); a signal threshold is set on  
109 reference pictures made from each pictures by RB25 and “sharpened hat” algorithms. After  
110 watershed, upper and lower limits are set to exclude debris and the number of viable and or  
111 dead (PI+) cells is calculated for each well.

112 The cells were manually examined in an inverted fluorescence microscope with ultraviolet  
113 excitation wavelength at 340–380 nm. Viable or necrotic cells were identified by intact nuclei  
114 with, respectively, blue (Ho) or pink (Ho plus PI) fluorescence. Apoptotic cells were detected  
115 by their fragmented nuclei which exhibited either a blue (Ho) or pink (Ho plus PI)  
116 fluorescence depending on the stage in the process (Fig 1B). In early apoptosis, only  
117 Hoechst 33342 will reach the nuclear material, whilst in a later phase PI will also penetrate  
118 the cells. In each condition and experiment, a minimum of 300 cells were counted.  
119 Percentages of living, apoptotic, and necrotic cells were expressed as mean ± SD.

## 120 **Detection of UCHL1 and GAD65**

### 121 **Detection antibodies**

122 UCHL1 was detected by a sandwich antibody couple consisting of a capture antibody,  
123 polyclonal goat anti-UCHL1 (Acris Antibodies, USA) raised against a peptide corresponding  
124 to amino acids 58-68) and rabbit polyclonal anti-UCHL1 detection antibody (Atlas Antibodies,  
125 Sigma Aldrich, USA), raised against a peptide corresponding to amino acids 74-219) [5].  
126 GAD65 was detected by previously described antibodies [17]. The detection antibodies were

127 biotinylated using EZ Link™ NHS-Biotin (Thermo Fisher Scientific Inc., USA) according to  
128 manufacturer's protocol.

### 129 **Cytometric bead array (CBA)**

130 The capture antibodies were covalently attached to CBA functional beads A4 and E7 (BD  
131 Biosciences, USA) with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate  
132 chemistry according to manufacturer's protocol. Functionalized beads were stored shielded  
133 from light at 4°C for no more than six months. Recombinant proteins were used to generate  
134 standard curves and quality controls were stored in single use aliquots at -80°C providing  
135 long-term stability (recombinant human UCHL1, Sigma Aldrich, USA and recombinant  
136 human GAD65, Diamed, Sweden). For a single measurement, 25 µL of sample (recombinant  
137 standard material or supernatant sample) was mixed with 25 µL of Assay Diluent (BD  
138 Biosciences, USA) and 20µL beads solution at 6000 beads per well in Capture Bead Diluent  
139 (BD Biosciences, USA). This was incubated in a U-shaped 96-well predilution plate overnight  
140 at 4°C, shaken and shielded from light. Hereafter, 20µL of a 5µg/mL solution of biotinylated  
141 detection antibody for UCHL1 and GAD65 was added to all wells without prior washing. After  
142 1 hour incubation at room temperature on the shaking plate, wells were transferred to  
143 Multiscreen® filtration plate (Merck KGaA, Germany) and washed 6 times with 150 µl of  
144 Wash Buffer (BD Biosciences, USA) using a Millipore Multiscreen (Perkin Elmer, USA)  
145 adaptor, followed by addition of 100 µL of Human Enhanced Sensitivity Detection Reagent  
146 (Part B) (BD Biosciences, USA). After incubation for one hour at room temperature, shaken  
147 and shielded from light, wells were washed 6 times and content was transferred to 1.5 mL  
148 microcentrifuge tubes for analysis on FACS Aria cell sorter (BD Biosciences, USA). Single  
149 beads were gated based on forward scatter, their intrinsic color code defined by  
150 Allophycocyanine (APC)-cyanine (CY7) dye couple after excitation by a 633 nm laser.  
151 Clustering parameters for the beads were defined by their forward and sidescatter properties  
152 and their APC and APC-Cy7 fluorescence excited by a 640 nm laser. Bound biotinylated  
153 detection antibody was quantified by quantification of streptavidin-bound phycoerythrin (PE)

154 fluorescence at 488 nm. Frequency distribution histograms of PE-fluorescence showed a  
155 single high peak in all samples and the median PE fluorescence of this peak was selected for  
156 quantification with Diva software (BD Biosciences, USA). Analytical limit of detection (LOD,  
157 mean of blanks + 3SD) in culture medium was 5.99 pmol/L for UCHL1 and 0.27 pmol/L for  
158 GAD65.

### 159 **Western Blotting**

160 Antibody dilution used for Western blotting on NuPAGE Novex 10% Bis-Tris Midi Gel  
161 (Thermo Fisher Scientific, USA): primary detection with polyclonal rabbit anti-UCHL1 (Aviva  
162 Systems Biology, San Diego, USA) at 1:1000 dilution and HRP-linked secondary anti-rabbit  
163 antibody (1:1000, GE Healthcare limited, UK). Intensities of bands were quantified by ImageJ  
164 software and normalized to beta-actin (rabbit anti-ACTB, 1:1000, Abcam, Cambridge, UK).

### 165 **In vitro human islet transplant samples**

166 Human islet cells were cultured for maximally 4 weeks in serum-free Ham's F10 medium and  
167 characterized for beta cell number, endocrine purity and viability before selection for clinical  
168 transplantation as described [4]. In this study, standardized islet grafts (n=14) sampled at the  
169 time of intra-portal transplantation were cultured for 24 hours in 24 well plates with 500  $\mu$ L  
170 serum-free Ham's F10 medium. Subsequently, cells and medium were collected, centrifuged,  
171 and supernatant from duplicate samples were combined. Human pancreatic islet graft  
172 characteristics are described in Table 2.

### 173 **In vivo human islet transplant plasma samples**

174 The UCHL1-GAD65 CBA assay was also applied to measure circulating UCHL1 and GAD65  
175 concentrations after islet transplantation: non-uremic T1D patients typically received intra-  
176 portal infusion of at least  $2 \cdot 10^6$  beta cells/kg bodyweight and plasma was sampled at various  
177 time points before and up to 24 hours after transplantation. Plasma was collected in K3-  
178 EDTA Monovette® tubes (Sarstedt) supplemented with 1% of a 0.12mg/mL solution of  
179 aprotinin (Stago) in 0.9% NaCl solution. Storage was done at -80°C after centrifugation for 15  
180 minutes at 1600g.

## 181 **Ethics Statement**

182 All experiments involving humans and animal models were approved by the medical ethics  
183 committee of Universitair Ziekenhuis Brussel and Vrije Universiteit Brussel (Commissie  
184 Medische Ethiek, O.G. 016, Reflectiegroep Biomedische Ethiek, Laarbeeklaan 101, B1090  
185 Brussels, Belgium, Email: [Commissie.ethiek@uzbrussel.be](mailto:Commissie.ethiek@uzbrussel.be) ). Use of animal and human  
186 tissues for in vitro biomarker validation was covered by B.U.N. 143201213515 for a project  
187 entitled “Identification and clinical validation of biological markers for beta cell death or  
188 dysfunction”, approval to G. Martens. Use of rat beta cells was covered by project reference  
189 12-274-2 entitled “Use of rats for preclinical transplant experiments”, approval to Prof. Dr. D.  
190 Pipeleers. Use of plasma samples obtained from human islet grafts recipients was covered  
191 by project reference 2005/136 prot, entitled “Long-term function of pancreatic beta cell  
192 allografts in non-uremic type 1 diabetic patients”. Use of human beta cells for in vitro  
193 research was covered by project reference CME 2005/118 and CME 2010/193 (approval to  
194 the UZ Brussel BetaCell Bank, B.U.N. 14320109289) and by project reference CME  
195 2009/018, entitled “platform for beta cell therapeutics” and CME 2009/017, entitled CME  
196 2009/017, approval to the JDRF Center for Beta Cell Therapy at Vrije Universiteit Brussel.

197 Human islet grafts that did not meet the criteria for clinical transplantation were provided for  
198 secondary in vitro use according to the Belgian law (December 19, 2008 – Wet inzake het  
199 verkrijgen en het gebruik van menselijk lichaamsmateriaal met het oog op geneeskundige  
200 toepassing op de mens of the wetenschappelijk onderzoek) along the principle of presumed  
201 consent, unless an express objection was made by the person or next of kin prior to the  
202 death. All organs are traceable through written forms (Eurotransplant Pancreas Report,  
203 Organ inspection record, donor feedback record, Beta Cell Graft Release Record, Request  
204 forms for human pancreatic tissue/cells ) archived at the BetaCell Bank at UZ Brussel.

205 All human islet graft recipients provided written informed consent to use blood samples,  
206 along predefined blood sampling algorithms reviewed by our medical ethics committee.  
207 Informed consent forms were made up in Dutch and French, and approved by the medical



208 ethics committee of Universitair Ziekenhuis Brussel and Vrije Universiteit Brussel, and  
209 consent forms archived by the UZ Brussel Clinical Trial Center.

## 210 **Statistical analysis**

211 Data were expressed as mean  $\pm$  SD and p-values were calculated with Mann-Whitney U-test  
212 using GraphPad Prism (GraphPad Software, USA). Statistical significance is denoted by  
213 \* $p < 0.05$  and \*\* $p < 0.0001$ .

## 214 **Results**

### 215 **Imaging-based measurement of rat beta cell death in vitro**

216 On day 0, 6000 single rat beta cells were seeded and exposed to two known inducers of  
217 apoptotic beta cell death [9], ER-stress inducer thapsigargin (0.2  $\mu$ M) and a cocktail of pro-  
218 inflammatory cytokines: TNF- $\alpha$ +IL-1 $\beta$ +IFN- $\gamma$  (all at 1ng/mL final concentration). Hydrogen  
219 peroxide (H<sub>2</sub>O<sub>2</sub>; 100 $\mu$ M final concentration), a potent oxidative inducer of necrotic rat beta  
220 cell death was used as positive control [18]. Cell death and viability were measured after 3  
221 and 6 days using an operator-dependent (manual) or -independent (semi-automated)  
222 microscopic method. In the semi-automated method, a whole well image was generated  
223 using a BD Pathway 435 platform as shown in Fig 1A, the total number of cells was counted  
224 by integrating Hoechst-positive events and the number of dead, propidium-iodide positive  
225 cells was calculated using BD Attovision software. These same wells were then subjected to  
226 manual counting of percentage necrotic, apoptotic and living cells as indicated in Fig 1B and  
227 1C. H<sub>2</sub>O<sub>2</sub> rapidly induced massive necrosis resulting in > 90% necrotic cells at day 3. On day  
228 3, the thapsigargin -and cytokine-toxicities became detectable with 36.0 (37.3) % and 45.9  
229 (41.9) % dead cells, respectively as detected by the semi-automated (manual) counting.  
230 However, this effect failed to reach statistical significance as compared to control cells 35.0  
231 (29.2)%. On day 6, thapsigargin and cytokines resulted in death of 82.9 (88.7) % and 67.1  
232 (63.7) % cells, respectively, and expectedly, the predominant modality of cell death was  
233 apoptosis (Fig 1E). The side-by-side comparison between automatic and manual counting of

234 cell death indicated overall an excellent agreement ( $R^2 = 0.956$ , Fig 1F) between semi-  
235 automated and manual methods, thus providing the required reference to investigate the  
236 added value of measuring discharged biomarkers as sensitive indicators of *in vitro* beta cell  
237 toxicity.

238

## 239 **Biomarker-based measurement of rat beta cell death in** 240 **vitro**

241 Loss of beta cell plasma membrane integrity results in the discharge of obligate intracellular  
242 proteins that, when sufficiently beta cell-selective, qualify as real-time indicators of this death  
243 process. Previous studies, using relatively insensitive immunoassays, indicated that GAD65  
244 and UCHL1 are extracellularly discharged under conditions of severe necrotic destruction [5,  
245 6]. Here, we investigated if a newly developed enhanced-sensitivity bead-based duplex  
246 assay for GAD65 and UCHL1 could also detect minor instances of apoptotic beta cell death  
247 *in vitro*, taking the imaging-based methods (Fig. 1) as reference. UCHL1 came out as best  
248 indicator: (i) Induced UCHL1 discharge by thapsigargin and cytokine-induced cell death was  
249 consistently ( $P < 0.05$ ) detected starting day 3. At this time point, GAD65 detected cytokine-  
250 but not thapsigargin-induced cell death (Fig. 2A); (ii) Stoichiometrically, extracellular UCHL1  
251 was 20 times more abundant than GAD65; (iii) the molar amount of UCHL1 discharged  
252 between day 3 and 6 showed an overall good agreement with the imaging-based increment  
253 of cell death during this time frame (Fig. 2B), while this was not the case for GAD65; (iv)  
254 UCHL1, was discharged early during the incubation, as occurs during  $H_2O_2$ -induced  
255 necrosis. It remained stable throughout the full 6 day incubation at  $37^\circ C$  while GAD65  
256 showed 30 % degradation between day 3 and day 6. This is in line with its reported  
257 thermolability [19], and was also confirmed in a stability experiment in which GAD65 was  
258 spiked in culture medium in absence of cells and maintained at  $37^\circ C$  for 3 days; this resulted  
259 in only 19% recovery (Supplementary Fig 1). Overall, the discharged amounts of both

260 UCHL1 and GAD65 showed a significant ( $P < 0.0001$ ) linear correlation to the number of dead  
261 beta cells, but expectedly this correlation was the strongest for UCHL1 (Fig. 2C).

262

263 Analysis of biomarker recovery revealed, however, one anomaly for UCHL1: since  
264 discharged UCHL1 is extracellularly stable (Fig. S1 and Fig. 2), the amount of released  
265 UCHL1 should be equivalent to the intracellular content of the disrupted beta cells. This was  
266 not the case: for each K dead beta cell, 9.5 fmol UCHL1 was recovered from the medium,  
267 well above the intracellular content measured by the same CBA-method in isolated cells  
268 (Table 1). This excessive recovery of UCHL1 *in vitro* was previously observed after severe  
269 necrosis by hydrogen peroxide [5]. It could not be explained by increased adaptive UCHL1  
270 biosynthesis under conditions of ER-stress (S2 Fig), leaving the option that it may results  
271 from an altered immune reactivity of UCHL1 during extracellular discharge, e.g. by loss of  
272 intracellular polymer structure [20]. This might explain higher observed intracellular UCHL1  
273 levels by denaturing methods such as Western blotting (Table 1).

<b>Table 1. Intracellular content of UCHL1 and GAD65</b>				
<b>UCHL1</b>	<b>RAT</b>		<b>HUMAN</b>	
<b>fmol/K</b>		<b>n</b>		<b>n</b>
CBA	1.1 (0.4-1.3)	4	0.8 (0.7-1.1)	5
Western Blot	2.0 (1.2-4.3)	4	9.5 (4.5-18.5)	8
<b>GAD65</b>	<b>RAT</b>		<b>HUMAN</b>	
<b>fmol/K</b>		<b>n</b>		<b>n</b>
CBA	3.0 (1.7-4.3)	3	1.1 (0.7-1.6)	4
Data represent median with IQR in parentheses				

274

275 **Biomarker-based measurement of human beta cell death in**  
276 **vitro**

277 Human beta cells, prepared for clinical transplantation, survive and function optimally when  
 278 cultured as endocrine aggregates, referred to as islet equivalents. Since aggregate  
 279 suspension cultures technically impede image-based measurement of beta cell death,  
 280 biomarker-based measurements represent a useful alternative. Therefore, we measured  
 281 UCHL1 and GAD65 discharge *in vitro* over 24h by standardized islet grafts (n=14) sampled  
 282 at the time of intra-portal transplantation, and studied statistical correlations to the graft  
 283 characteristics shown in Table 2. The grafts showed a good overall viability and a low level of  
 284 contamination with exocrine acinar cells, as measured by electron microscopy, and  
 285 state-of-the-art endocrine purity (95%CI) with 31% (19-35)% insulin-positive cells. The  
 286 amount of UCHL1 discharged *in vitro* correlated significantly both to the total percentage of  
 287 dead cells, and to the number of beta cells. Also for GAD65, a correlation could be found  
 288 with the number of beta cells, but this correlation was less strong compared to UCHL1 and  
 289 was also seen with total cell number (Table 2).

<b>Table 2. Human pancreatic graft characteristics</b>				
Cellular composition (% total)			<b>Correlation with UCHL1</b>	<b>Correlation with GAD65</b>
Electron microscopy	Dead	8 (5-10)	<b>0.0113</b>	<b>ns</b>
	Exocrine	4 (1-6)	<b>ns</b>	<b>ns</b>
	Non Granulate	54 (45-65)	<b>ns</b>	<b>ns</b>
	Endocrine	34 (20-44)	<b>ns</b>	<b>ns</b>
ICC	Insulin <sup>+</sup>	31 (19-35)	<b>ns</b>	<b>ns</b>
	Glucagon <sup>+</sup>	9 (5-17)	<b>ns</b>	<b>ns</b>
Cell number (k/well)				
	Total cell number	304 (216-413)	<b>ns</b>	<b>0.0004</b>
	Beta cell	75 (59-112)	<b>0.0005</b>	<b>0.0130</b>

	number			
Data represent median for 14 grafts with IQR in parentheses				
Non parametric Spearman correlation				

290

## 291 **Failure of UCHL1 to detect human beta cell death in vivo**

292 GAD65 was previously validated as real-time indicator of human beta cell death after islet  
293 transplantation [21]. UCHL1 could be confirmed as *in vivo* indicator of beta cell death in  
294 streptozotocin-injected rodents, albeit variably and hampered by fast hepatic biomarker  
295 clearance [5]. We tested the duplex UCHL1-GAD65 CBA assay in GAD65-autoantibody  
296 negative islet graft recipients: while an acute plasma GAD65 surge could consistently be  
297 detected, UCHL1 remained undetectable, both after the standard implantation in the liver  
298 (Fig 3), as after experimental implantation into the omentum, indicating that the superior *in*  
299 *vitro* performance of UCHL1 as compared to GAD65 is not translated into clinical diagnostic  
300 use.

301

## 302 **Discussion and Conclusions**

303 Here we report a novel method for *in vitro* analysis of rodent and human beta cell toxicity  
304 using a sensitive duplex UCHL1-GAD65 CBA assay. The quantification of discharged beta  
305 cell-biomarkers has several technical advantages for *in vitro* toxicological analysis of beta  
306 cells, as compared to microscopic quantification of vital dyes or conventional tetrazolium dye-  
307 based (MTT) assays: (i) by targeting endocrine-restricted biomarkers, it can specifically  
308 quantify beta cell injury in mixed cultures or impure islet fractions. It performs equally well in  
309 adherent and suspension cultures, and unlike MTT, independently of the metabolic  
310 phenotype of the cells; (ii) by using an enhanced-sensitivity bead-based immunoassay  
311 approach [17], it achieves sensitivities that could, in our hands, not be reached by MTT  
312 assays on the small numbers of cells used in our experimental design, achieving even

313 superior sensitivity than the microscopic measurement of vital dyes; (iii) moreover, soluble  
314 markers allow repeated sampling of the same wells and, unlike MTT and microscopy, does  
315 not represent endpoint studies, thus allowing kinetic time-resolved analyses, and interim  
316 sampling for quality control purposes, and finally (iv) the assay allows upscaling to high-  
317 throughput formats on widely available immunoassay platforms.

318 *In vitro*, UCHL1 appears superior to GAD65 as indicator of beta cell injury: UCHL1 showed a  
319 better thermo-stability and outperformed GAD65 to detect early stages of beta cell apoptosis,  
320 although its unexplained excessive immuno-recovery still necessitates the use of internal  
321 controls. We repeatedly observed a 3-7 fold higher recovery of immuno-detectable UCHL1  
322 than could be expected based on measurement of intracellular content. We hypothesize that  
323 this behavior is explained by the loss of UCHL1 multimeric structure [23] after extracellular  
324 discharge, inducing a higher accessibility of the UCHL1 epitopes targeted by our sandwich  
325 immunoassay on the UCHL1 monomers. This behavior requires specific attention, since  
326 UCHL1 is increasingly used in patients to quantify neuronal damage [23, 24]. UCHL1 is  
327 reported as one of the most abundant proteins in neurons [23]. In human beta cells it ranks  
328 among the higher-abundance core proteome detectable by LC-tandem MS [5, 25, 26]. Its  
329 neuroendocrine-selectivity, combined with its high cellular abundance, favor the use of  
330 UCHL1 as biomarker to detect neuronal or neuroendocrine cell death *in vivo*. Several groups  
331 reported that intact UCHL1 protein can pass the blood brain barrier and become detectable  
332 in the circulation after acute traumatic or ischemic brain injury [24]. Using our sandwich  
333 immunoassay, we could detect beta cell-derived UCHL1 in the plasma after streptozotocin-  
334 induced synchronous and massive induction of beta cell necrosis in rats [5]. Here, we show  
335 that despite its excellent *in vitro* behavior, the use of UCHL1 failed the translation to the  
336 transplantation clinics. In human islet graft recipients, UCHL1 could not be detected after  
337 intra-hepatic nor after intra-omental transplantation, although we expected detectable levels  
338 as estimated by extrapolation of its *in vitro* discharge compared to GAD65. We hypothesize  
339 that the failure of UCHL1 might be attributed to its fast hepatic clearance [5] or, loss of its  
340 immune recognition due to polymerization or physiological binding of ubiquitinated-proteins in

341 its catalytic center. If the latter is true, this would also predict variability of immuno-detectable  
342 UCHL1 in other models (e.g. traumatic brain injury), and caution in the use of UCHL1 as  
343 quantitative indicator of neuroendocrine cell death.

344 In conclusion: the use of soluble biomarkers represents a fast, selective and sensitive  
345 method for beta cell toxicity profiling *in vitro*. UCHL1 is superior to GAD65 *in vitro* but not *in*  
346 *vivo*.

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357

## 358 **Figure legends**

359

360 **Fig 1. Imaging-based measurement of rat beta cell death *in vitro*.** Representative  
361 Hoechst-Propidium iodide vital staining of single rat beta cells following exposure to TNF- $\alpha$  +  
362 IL-1 $\beta$  + IFN- $\gamma$  for 6 days. (A) Whole-well image on BD-Pathway 435, segmentation using  
363 Image Processing Lab software. Hoechst positive cells in blue and propidium iodide positive  
364 cells in red. (B) Single rat beta cells, representative image for manual counting. Scale bar  
365 indicates 100 $\mu$ m. (C) Inset shows detail of panel B with viable (blue), necrotic (red) and live

366 (pyknotic nucleus, blue) and dead (fragmented nucleus, red) apoptotic cells (D) Number of  
367 viable/dead cells counted automatically with Attovision software for the different conditions  
368 over time (day 3 and day 6). Bars represent mean  $\pm$  SD (n=2). (E) Percentage of cells  
369 accounted to viable, necrotic or apoptotic with manual counting for the different conditions  
370 over time (day 3 and day 6). Bars represent mean  $\pm$  SD (n=2-4). (F) Correlation between  
371 number of dead cells counted automatically with Attovision software and percentage of dead  
372 cells (necrotic and apoptotic) by manual counting. A positive correlation was found with  
373 Spearman  $r = 0.960$ ,  $R^2 = 956$ ,  $P < 0.0001$ . Dotted lines indicate 95% prediction interval.

374 **Fig 2. Biomarker-based measurement of rat beta cell death *in vitro*.** (A) UCHL1 and  
375 GAD65 protein discharged from beta cells following exposure to thapsigargin (0.2 $\mu$ M),  
376 cytokines TNF- $\alpha$  + IL-1 $\beta$  + IFN- $\gamma$  (all 1 ng/mL) or hydrogen peroxide H<sub>2</sub>O<sub>2</sub> (100 $\mu$ M) compared  
377 to control (culture medium) at day 3 and day 6. \*  $p < 0.05$  versus control, Mann Whitney test  
378 (B) Difference in cell death (%), UCHL1 (fmol) and GAD65 (fmol) between day 6 and day 3  
379 expressed as delta values. (C) Correlation between UCHL1 and GAD65 (fmol) and % dead  $\beta$   
380 cells as found by manual counting. A positive correlation was found for both proteins with  
381 Spearman  $r = 0,912$ ,  $P < 0.0001$  and Spearman  $r = 0.771$ ,  $P < 0.0001$  respectively.

382 **Fig 3. Biomarker-based measurement of human beta cell death *in vivo*.** Plasma samples  
383 from islet transplantations (Tx) (n=5) were analyzed for their GAD65 ( $\blacktriangle$ ) and UCHL1 ( $\blacksquare$ )  
384 concentration. Venous blood samples were taken before Tx (Pre) and early after Tx at 5-15-  
385 30-60 minutes.

## 386 **Supplementary figures legends**

387 **Supplementary Fig.1 : Stability of recombinant and endogenous proteins.** UCHL1  
388 (blue) and GAD65 (red) spiked in culture medium and stored at 37°C and 5%CO<sub>2</sub> for 3 to 6  
389 days.

390



391 **Supplementary Fig 2: No adaptive UCHL1 biosynthesis under conditions of ER-stress.**

392 Rat beta cell aggregates were exposed to thapsigargin (0.2 $\mu$ M) for 24 hours. Cell lysate was  
393 compared to control condition (aggregates exposed to culture medium) and analysed with  
394 Western Blotting and with duplex UCHL1-GAD65 cytometric bead assay (A). Both analysis  
395 revealed no significant difference between rat aggregates exposed to stress and control  
396 condition. (B) UCHL1 and GAD65 mRNA data from previously published dataset [27]. RNA  
397 sequencing was used to identify transcripts, including splice variants, expressed in human  
398 islets under control conditions or following exposure to the pro-inflammatory cytokines IL-1 $\beta$   
399 and IFN- $\gamma$ . This revealed no significant difference between human islets exposed to  
400 cytokines or control condition.

401

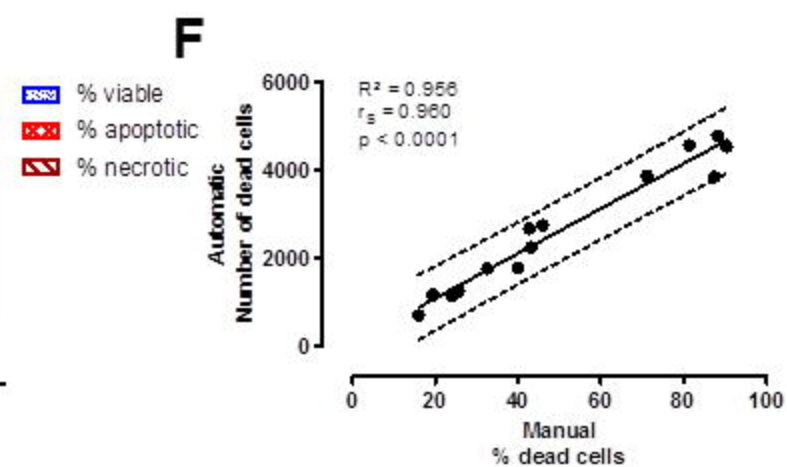
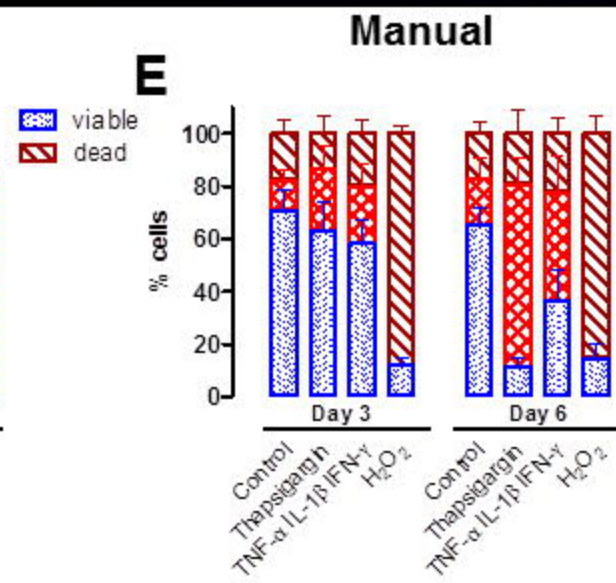
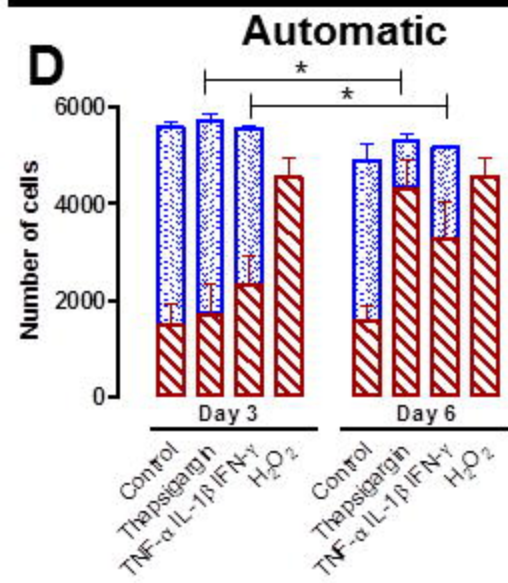
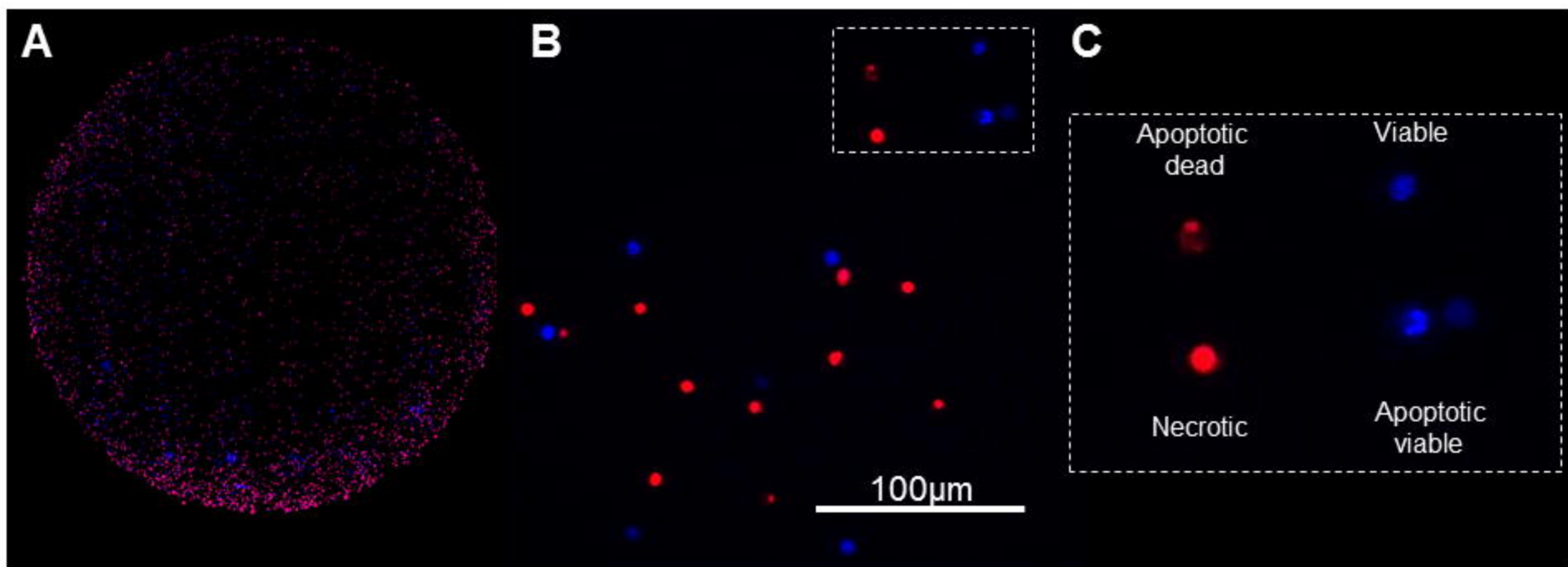
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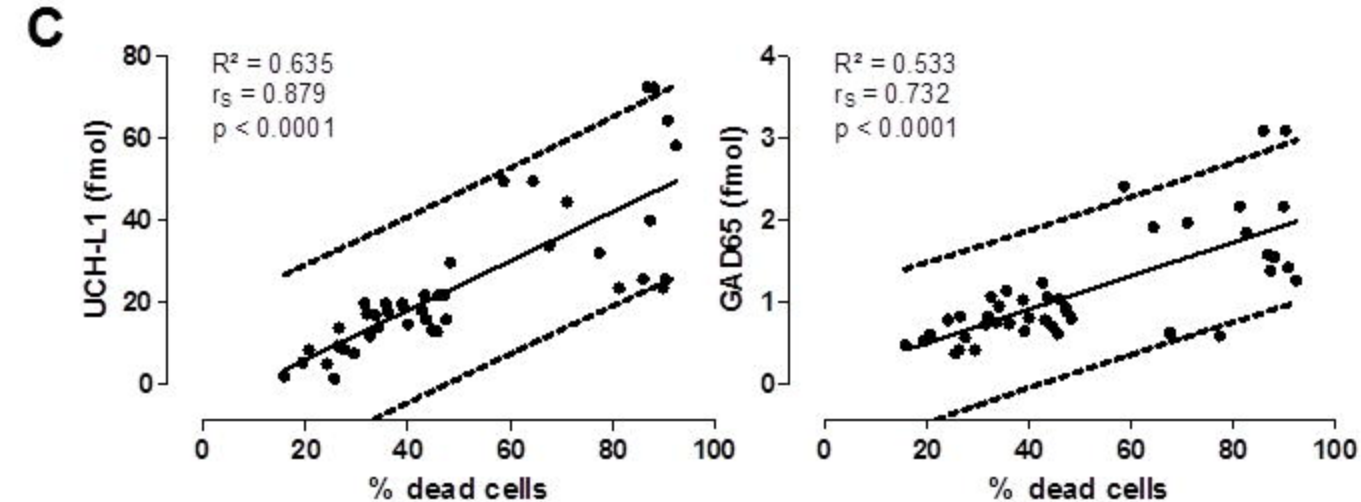
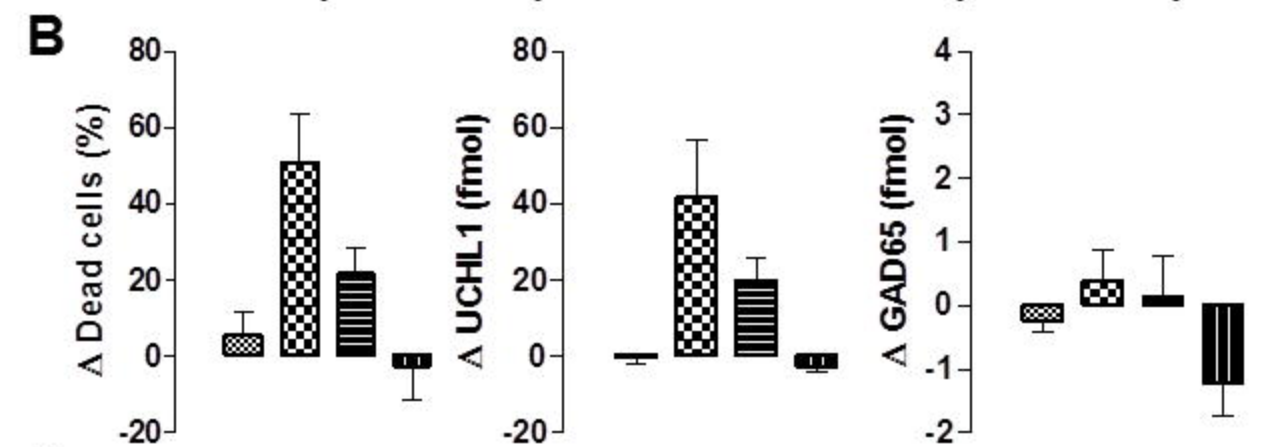
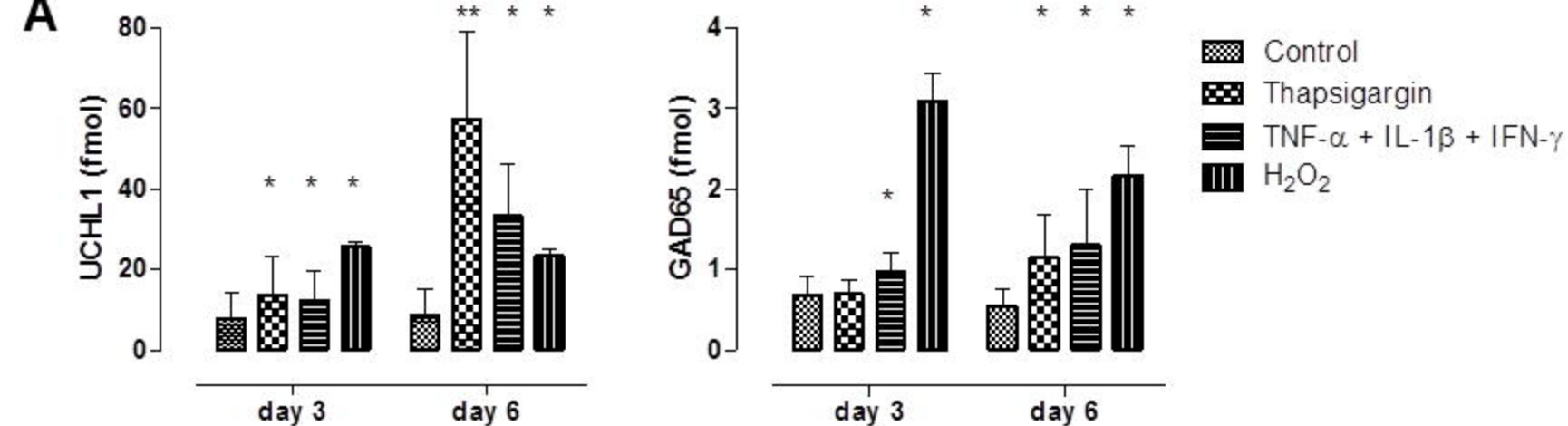
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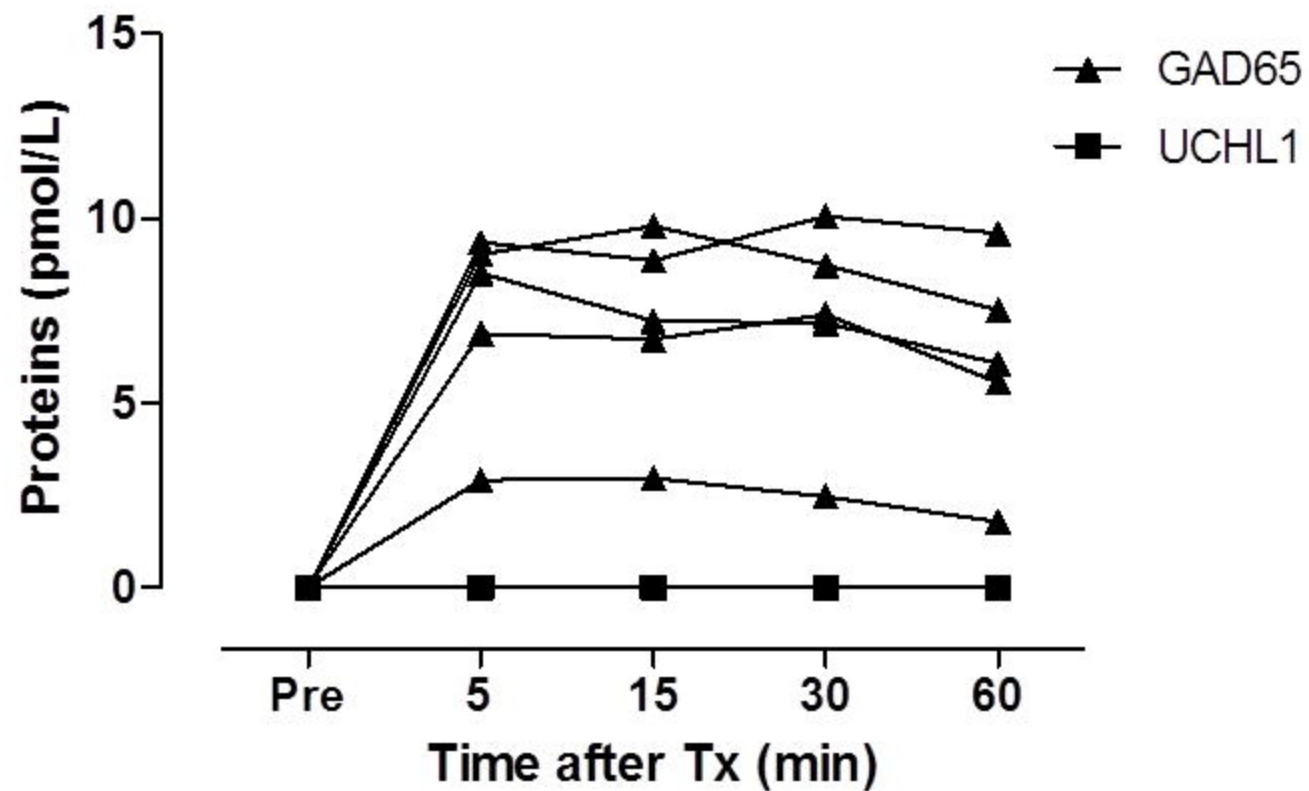
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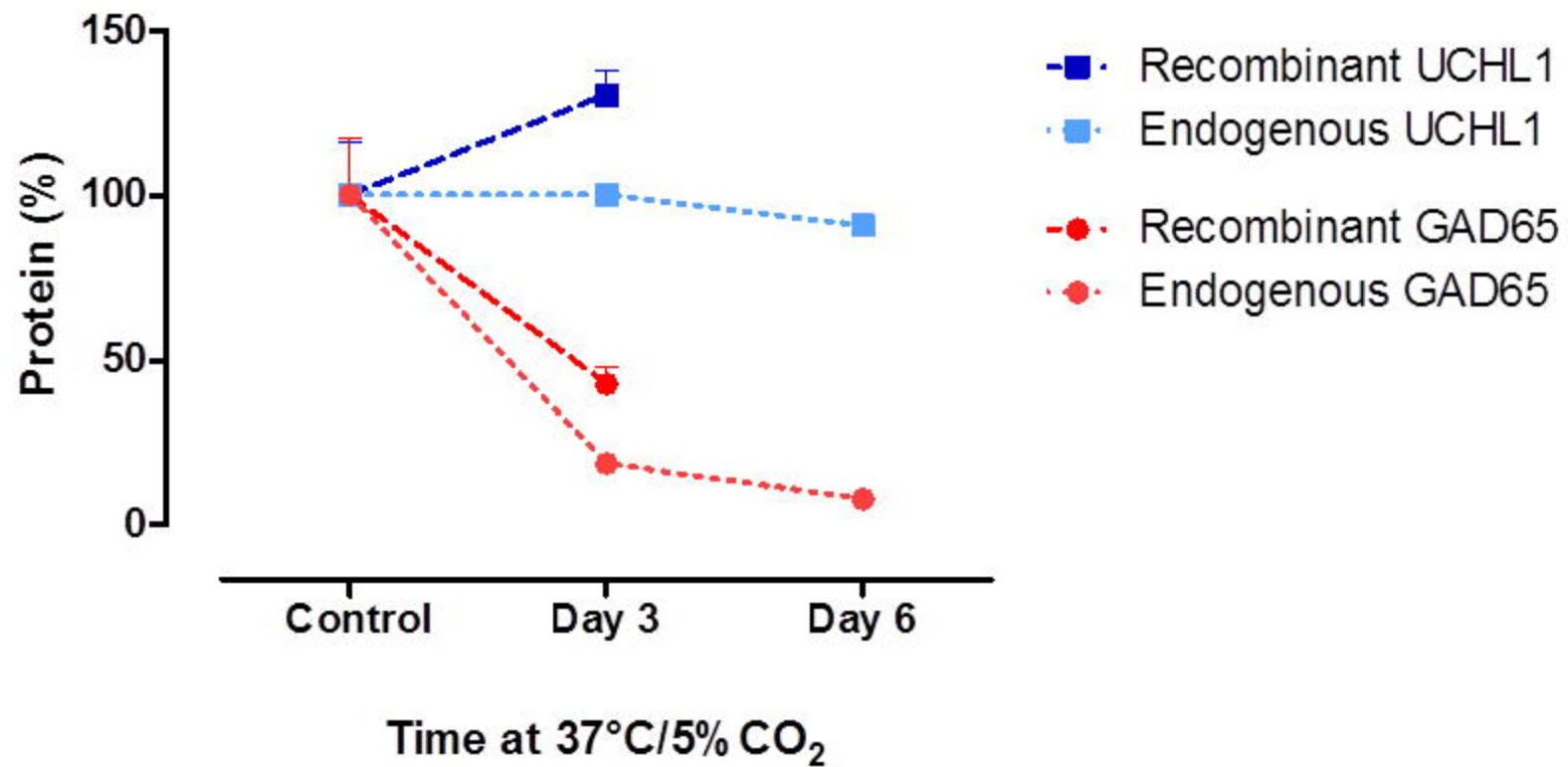
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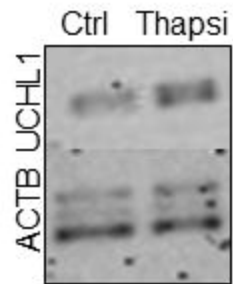










**A** Western Blot:CBA:

CBA	fmol/K
Ctrl	3,3
Thapsi	3,4

**B** RNA sequencing: