

## Di and tripeptides from marine sources can target adipogenic process and contribute to decrease adipocyte number and functions

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1 **Di and tripeptides from marine sources can target adipogenic process and**  
2 **contribute to decrease adipocyte number and functions**

3

4 Ben Henda Yesmine<sup>a</sup>, Laamari Mariem<sup>a</sup>, Lanneluc Isabelle<sup>a</sup>, Travers Marie Agnès<sup>b</sup>, Agogue  
5 Hélène<sup>c</sup>, Arnaudin Ingrid<sup>a</sup>, Bridiau Nicolas<sup>a</sup>, Maugard Thierry<sup>a</sup>, Piot Jean-Marie<sup>a</sup>, Sannier  
6 Frédéric<sup>a</sup> and Bordenave-Juchereau Stéphanie<sup>a</sup> \*

7 <sup>a</sup>Université de La Rochelle, Laboratoire LIENSs UMR CNRS-7266, Approches Moléculaires,  
8 Environnement Santé, site Marie Curie, UFR Sciences Technologies Santé, F-17042 La  
9 Rochelle cedex-1.

10 <sup>b</sup> Ifremer, SG2M-LGPMM, Laboratoire de Génétique et Pathologie des Mollusques Marins  
11 Avenue de Mus de Loup, 17390 La Tremblade, France.

12 <sup>c</sup> Molecular Core Facility, Laboratoire LIENSs UMR7266 CNRS-Université de La Rochelle,  
13 2 rue Olympe de Gouges, 17000 La Rochelle France.

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15

16 \*Corresponding author

17 Email: sbordena@univ-lr.fr

18 Tel: +33546458562

19 Fax: +33546458265

20

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22 **Abstract**

23 The effect of 11 marine-derived cryptides was investigated on proliferation, differentiation  
24 and maturation of human white pre-adipocytes (HWP). They were all formerly identified as  
25 potent Angiotensin-Converting-Enzyme inhibitors. Val-Trp (VW), Val-Tyr (VY), Lys-Tyr  
26 (KY), Lys-Trp (KW), Ile-Tyr (IY), Ala-Pro (AP), Val-Ile-Tyr (VIY), Leu-Lys-Pro (LKP),  
27 Gly-Pro-Leu (GPL), Ala-Lys-Lys (AKK) and Val-Ala-Pro (VAP) were previously found in  
28 fish products and co-products as well as other marine resources like wakame. Treatment with  
29 AP, VAP and AKK greatly affected viability of HWP during the proliferation period while  
30 KW and VW treatment reduced the number of viable cells during the differentiation stage. A  
31 GPL and IY incubation during the differentiation stage allowed the decrease of their final  
32 lipid content, of the GPDH activity and of the mRNA level of adipocytes markers (aP2,  
33 GLUT4, LPL and AGT). Moreover, a down regulation of both PPAR $\gamma$  and C/EBP $\alpha$   
34 expression, two key regulators of adipogenesis was observed. These findings indicate that  
35 small bioactive peptides from marine protein hydrolysates can target adipogenesis and thus  
36 could regulate energy metabolism disorders.

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41 Keywords: Bioactive peptides, Obesity, Human white pre-adipocytes, proliferation,  
42 differentiation, adipocyte differentiation markers.

43

## 44 **1. Introduction**

45 Around 1.6 billion adults (over the age of 15 years) are considered to be over weighted (with  
46 a body mass index (BMI) between 25 and 30 kg/m<sup>2</sup>) and 400 million are considered obese  
47 (with a BMI  $\geq$  30 kg/m<sup>2</sup>) (Stothard, Tennant, Bell, & Rankin, 2009). These data demonstrate  
48 the urgency to establish effective strategies for prevention and treatment of obesity. Among  
49 ways to reduce obesity, the decrease of energy/food intake and the increase of energy  
50 expenditure (Park, Jeon, Kim, & Han, 2013) are of importance but targeting adipocyte  
51 physiology and cell cycle could also be a solution. Indeed, recent studies have examined how  
52 far inhibition of preadipocyte proliferation, differentiation, lipogenesis or promotion of  
53 lipolysis and fat oxidation (Wang, & Jones, 2004) can reduce obesity. Obesity is characterized  
54 at the cellular level by an increase in the number and size of adipocytes originating from  
55 fibroblastic pre-adipocytes in adipose tissues (Furuyashiki et al., 2004). Pre-adipocytes, which  
56 can then be differentiated into mature adipocytes and modulate body fat mass, play a key role  
57 in obesity (Park et al., 2013).

58 At a molecular level, differentiation is a well-organized program characterized by the loss of  
59 pre-adipocyte markers and sequential changes in the expression of general and adipocyte-  
60 specific genes that determine the specific adipocyte phenotype (Catalioto, Maggi, & Giuliani,  
61 2009). These changes in gene expression during differentiation program are induced by a  
62 coordinated expression and action of transcription factors. Consequently, early, intermediate  
63 and late mRNA and protein markers can be detected and finally triglycerides (TG)  
64 accumulation can be observed.

65 Moreover, mature adipocytes express all components of the renin-angiotensin system (RAS),  
66 including angiotensinogen (AGT), the sole precursor of Angiotensin II (Ang II), type 1 (AT1)  
67 and type 2 (AT2) angiotensin receptors as well as the angiotensin peptide forming enzymes:  
68 renin, angiotensin-I-converting enzyme (ACE), and chymase (Engeli, Negrel & Sharma,

69 2000). This local RAS exerts important auto/paracrine functions in modulating lipogenesis,  
70 lipolysis and adipogenesis (Massiera et al., 2001). Ang II, a potent vasoconstrictor and key  
71 player in hypertension, increases lipogenesis and TG accumulation in 3T3-L1 pre-adipocytes  
72 and human adipocytes (Jones, Standridge, & Moustaid, 1997). In addition, Crandall et al.  
73 (1999) observed that the stimulation of human pre-adipocytes with Ang II resulted in an  
74 acceleration of the G1-phase of the cell cycle and an increased expression of the cell cycle  
75 regulator *cyclin D1*. This suggests that Ang II can contribute to the growth and the  
76 development of adipose tissues. Thus blockade of the RAS and its main enzyme, ACE, may  
77 be of particular benefit in the management of obesity probably by reducing adipose size and  
78 decreasing functional adipocytes number. ACE- inhibitors (ACEI) may thus contribute to  
79 attenuate obesity by acting on adipocyte life cycle.

80 Since synthetic ACE inhibitors have often side effects, dietary bioactive peptides able to  
81 inhibit ACE could be interesting alternatives in the management of obesity.

82 We previously demonstrated that incubation of human adipocytes with a goat whey  
83 hydrolysate containing ACEI peptides decreased the proliferation of pre-adipocyte and  
84 reduced the lipid content of mature adipocytes (Hammé, Sannier, Piot, & Bordenave-  
85 Juchereau, 2010). The aim of this current work is to assess whether cryptides, bioactive  
86 peptides hidden within the sequence of a parent protein, already identified as potent ACEI can  
87 act on adipogenesis. These cryptides were selected in marine products such as fish, seaweed,  
88 and shellfish for their previously high inhibitory ACE potency, as demonstrated in  
89 administration studies with spontaneously hypertensive rats (SHR) (Ono, Hosokawa,  
90 Miyashita, & Takahashi, 2003). They also were chosen for their shortness giving them the  
91 opportunity to penetrate in tissue or cells and to resist to endogenous proteases. They can be  
92 found in sea bream, sardine or chicken indicating that these sequences are ubiquitous.

93 We investigated the ability of 6 dipeptides (Val-Trp (VW), Val-Tyr (VY), Lys-Tyr (KY),  
94 Lys-Trp (KW), Ile-Tyr (IY), Ala-Pro (AP)) and 5 tripeptides (Val-Ile-Tyr (VIY), Leu-Lys-Pro  
95 (LKP), Gly-Pro-Leu (GPL), Ala-Lys-Lys (AKK), Val-Ala-Pro (VAP)) to act on adipocyte  
96 development. A quite high concentration of 100 $\mu$ M was chosen for all peptides tested since it  
97 exhibited a noticeable effect on adipocytes development. Viability was measured after  
98 proliferation and differentiation of adipocytes. The regulatory effect of these peptides on the  
99 differentiation stage was analyzed by measuring the mRNA expression changes of adipocyte  
100 differentiation markers as well as assessing GPDH activity. Effect of peptides on lipid  
101 accumulation was also monitored after various times of exposition.

102 **2. Material and methods**

103 **2.1. Material**

104 Human white pre-adipocyte (HWP) were purchased as cryopreserved pre-adipocytes from  
105 PromoCell (Heidelberg, Germany) and originated from subcutaneous white adipose tissue  
106 obtained from female/62 years/Caucasian. The growth and differentiation media for pre-  
107 adipocytes, the nutrition medium for adipocytes and supplements for each medium were  
108 purchased from PromoCell.

109 The antibiotic solution containing Penicillin 10,000 U / ml and Streptomycin, 10 mg / ml was  
110 from PAN Biotech. GmbH (Aidenbach, Germany).

111 MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazoliumbromide), DMSO, Caffeine, Oil red  
112 O stain and Bradford reagent were from Sigma–Aldrich (Saint-Quentin Fallavier, France).

113 Synthetic peptides were purchased at >90% of purity from GeneCust (Dudelange,  
114 Luxembourg). Purity of each peptide was checked after solubilisation in adequate buffer and  
115 their molecular mass and aminoacid sequence were confirmed by HPLC ESI-MS.

116 **2.2. Cell Culture**

117 HWP cells were cultured according to the manufacturer's instructions with slight  
118 modifications. Briefly, cells not exceeding 4 passages were cultured at 37 °C in a humidified  
119 5% CO<sub>2</sub> atmosphere and grown in a preadipocyte Growth Medium (GM) with 1% antibiotics  
120 (proliferation stage).

121 For adipocyte differentiation, confluent cultures (Day 0) were stimulated to differentiation  
122 with pre-adipocyte differentiation medium (DM) containing d-Biotin 8 µg/ml, insulin 0.5  
123 µg/ml, dexamethasone 400 ng/ml, isobutylmethylxanthine 44 µg/ml, L-thyroxine 9 ng/ml and

124 ciglitazone 3µg/ml. After 4 days, medium was changed to the adipocyte nutrition medium  
125 (NM) containing Fetal Calf Serum 3%, d-Biotin 8µg/ml, insulin 0.5 µg/ml and  
126 dexamethasone 400 ng/ml, Cells were cultured in nutrition medium for further 4, 8 or 12 days  
127 according the test. By day 16, cells contained large lipid droplets and were considered as  
128 mature adipocytes.

129 Cryptides, dissolved in dimethyl sulfoxide (stock solution, 10<sup>-2</sup>M), were added at a final  
130 concentration of 100 µM to GM or DM and/or NM in order to observe their effects. Each  
131 medium was replaced every 2 days by fresh medium containing cryptides.

### 132 **2.3. MTT assay**

133 To detect the effect of marine cryptides on the viability of HWP during proliferation stage,  
134 HWP were seeded in 96-well culture plates at a density of 5000 cells/well and cultured in GM  
135 with or without 100 µM of marine cryptides. After 24 h or 72 h of treatment, 25µl MTT  
136 (5mg/mL) were added to each well. The plates were incubated at 37°C for 4 h, followed by  
137 the addition of DMSO (200 µl/well), and incubated at 37°C for 10 min. Optical density (OD)  
138 was measured at 570 nm using UV spectrometer Devices versa Max microplate reader  
139 (Molecular devices, California, United States). Non treated-cells were used as control.

140 To detect the effect of some cryptides on the viability of HWP cells during differentiation  
141 stage, cells were cultured with 100 µM of marine cryptides from day 0 to day 12. MTT assay  
142 was carried out on days 3, 6, 9 and 12 using the method described above.

### 143 **2.4. Oil-Red-O staining and quantification of lipid accumulation**

144 Lipid accumulation was estimated in mature adipocytes, cultured in 24-well plates at a density  
145 of 10<sup>4</sup>cells/ml. On day 16, medium was removed and cells were washed twice with PBS,  
146 fixed 1h with 500 µl 4% formaldehyde in PBS at room temperature, rinsed with 500 µl 60%



147 2-propanol and dried for 5min. Cells were stained with 200 µl Oil-Red-O solution (Oil-Red-O  
148 stock solution (5mg/ml in pure isopropanol): water=3:2)) at room temperature for 10 min.  
149 Then the Oil-Red-O solution was removed and cells were washed 4 times with distilled water  
150 to remove excess of stain. Images were obtained using Eclipse TS100 inverted microscope  
151 (100X) equipped for phase-contrast microscopy (Nikon, Yokohama, Japan).

152 For quantification, the stained oil droplets were dissolved in 750 µl isopropanol for 10 min  
153 and the lipid content evaluated by measuring the optical absorbance at 540 nm using Fluostar  
154 Omega spectrofluorometer (BMG LABTECH GmbH, Ortenberg, Germany).

### 155 ***2.5. Quantification of the GPDH activity***

156 HWP were seeded in 24-well plates, grown to confluence and treated during the  
157 differentiation period (from day 0 to day 8) with 100µM of cryptides (treated cells). A control  
158 with medium only was included in the study. On day 8, cells were washed twice with PBS,  
159 then enzyme extraction buffer was added to induce cell lysis, and cells extracts were collected  
160 by scraping. After centrifugation at 10,000 rpm for 5 min at 4°C, the glycerol-3-phosphate  
161 dehydrogenase (GPDH) specific activity of supernatant was measured with the GPDH  
162 Activity Assay Kit (TaKaRa Bio Inc., Shiga, Japan). GPDH Protein concentration was  
163 measured using the Bradford protein assay (Bradford, 1976). Values for GPDH activity were  
164 expressed as a proportion of control (100%).

### 165 ***2.6. Real time PCR***

166 HWP were seeded at 10<sup>5</sup> cell /ml in six well plates, grown to confluence, and then treated with  
167 100µM of cryptides during differentiation period (from day 0 to day 8). A control with  
168 medium only was included in the study. On day 8, cells were collected. Total RNA was  
169 extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to  
170 the manufacturer's instructions. The RNA samples were treated using the Ambion DNA-free

171 kit (Life Technologies, Carlsbad, USA). RNA was quantified at 260 nm using NanoDrop  
172 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA), and sample integrity was  
173 checked on 1% agarose gel electrophoresis.

174 Reverse-transcription of RNA (500 ng) was carried out using the Cloned AMV First-Strand  
175 cDNA Synthesis Kit (Invitrogen, Carlsbad, USA), with random primers following the  
176 manufacturer's instructions.

177 A real time PCR was then performed on cDNA samples to evaluate the mRNA expression of  
178 human genes PPAR $\gamma$ , C/EBP $\alpha$ , aP2, GLUT4, LPL and AGT. Human  $\beta$ -actin was used as an  
179 endogenous reference.

180 Reactions were run on an Mx3000P Real-Time Thermocycler (Stratagene, La Jolla, CA,USA)  
181 using Brilliant III Ultra-Fast SYBR Green kit (Applied Biosystems, Foster City, CA, USA)  
182 with the following cycling parameters: 1 cycle at 95°C for 3 min followed by 40 cycles at  
183 95°C for 10s and 60°C for 20 s. Each reaction was performed in duplicate in a final volume of  
184 20 $\mu$ l containing 5 $\mu$ L cDNA (1/20 dilution), 2 $\times$  Brilliant III Ultra-Fast SYBR Green Master  
185 Mix (10 $\mu$ L), and with an optimal concentration of gene specific forward and reverse primers  
186 (400 nM). The primer (Eurofins MWG Operon, Ebersberg, Germany) sequences are  
187 presented in table 1.

188 Relative quantification values were expressed using the  $2^{-\Delta\Delta C_t}$  method: the amount of target  
189 message (PPAR $\gamma$ , C/EBP $\alpha$ , aP2, GLUT4, LPL, or AGT in mRNA treated adipocytes) was  
190 normalized to the internal reference ( $\beta$ -actin) and compared to the calibrator (PPAR $\gamma$ ,  
191 C/EBP $\alpha$ , aP2, GLUT4, LPL, or AGT in untreated adipocytes).

192 The primers were validated by testing PCR efficiency using standard curves ( $95\% \leq$  efficiency  
193  $\leq 105\%$ ). PCR product specificity was evaluated by generating a dissociation curve following

194 the manufacturer's recommendations. All results were obtained from at least three  
195 independent experiments.

## 196 **2.7. Statistical Analysis.**

197 Data are presented as means  $\pm$  standard error. Statistical analyses were conducted using  
198 software Origin 6.0. A significant difference from the control group was analyzed with an  
199 unpaired Student *t* test. *P* values  $<0.05$  data were considered significantly different from those  
200 of the control.

201

## 202 **3. Results**

### 203 **3.1. Effect of marine cryptides on the viability of HWP cells during proliferation and** 204 **differentiation**

205 To detect the effect of marine cryptides on viability of HWP during the proliferation stage,  
206 cells were treated with 100  $\mu$ M of VW, KW, IY, KY, VIY, VY, LKP, GPL, AKK, AP, and  
207 VAP. MTT analysis was carried out after 24 h (Figure 1A) and 72 h (Figure 1B) of treatment.  
208 As shown in Figure 1A; AP, VAP and AKK affected cell viability significantly ( $p < 0.01$ ). VAP  
209 exhibited the greatest cell viability inhibition with a decrease of viability of  $59.3 \pm 7.3\%$  and  
210  $81.1 \pm 4.3\%$  after 1 and 3 days of treatment respectively when compared to controls. AP and  
211 AKK decreased viability by  $46.4 \pm 3.9\%$  and  $24.5 \pm 5.3\%$  and by  $55.8 \pm 4.5\%$  and  $22.8 \pm 4.3\%$ ,  
212 respectively, after 1 and 3 days of incubation while other cryptides tested showed no  
213 significant effect ( $p > 0.05$ ) on the HWP viability after 1 and 3 days of treatment during the  
214 proliferation stage. After 24 h, differences among effective peptides was significant between  
215 AKK and AP, VAP and AP, but not between AKK and AP. After 72 h, all values obtained  
216 after treatment with AKK, AP and VAP were significantly different.

217 Peptides exhibiting no anti-proliferative action during growth stage were then added in HWP  
218 medium during the differentiation period. 100  $\mu$ M of VW, VY, IY, KY, VIY, KW, GPL and  
219 LKP were added on cells and MTT analysis was carried out after 3, 6, 9 and 12 days during  
220 the differentiation stage. The cell viability was not affected after 72 h except in the presence  
221 of VW and KW which were found to reduce significantly ( $p<0.05$ ) the number of viable cells  
222 when compared with control (Figure 1C). For example, after 12 days, VW and KW decreased  
223 cell viability by  $32.8\pm 5.1\%$  ( $p<0.05$ ) and  $26.8\pm 4.3\%$  ( $p<0.05$ ) respectively.

### 224 ***3.2. Effect of marine cryptides on residual lipid content of mature adipocytes***

225 The inhibitory effect of cryptides on the final lipid content of mature adipocytes was studied,  
226 using those which did not exhibit adverse effect on adipocyte viability (GPL, IY, VIY, KY,  
227 VY and LKP). 100  $\mu$ M of cryptides or 100  $\mu$ M caffeine (chosen as a lipolytic reference  
228 molecule) was added to the culture medium. Figure 2A, B, C, D show the staining observed  
229 and Fig. 3, the quantitative results of mature adipocytes at day 16. As expected, the staining of  
230 HWP cultured in DM and NM without treatment revealed plenty of lipid droplets, indicating  
231 an almost total differentiation (Figure 2A).

232 Cells grown in DM and NM in presence of GPL (Figure 2B), IY (Figure 2C) or caffeine  
233 (Figure 2D), showed less oil droplets than the control cells indicating an inhibition of lipid  
234 filling when cells were treated with these cryptides. GPL treatment showed the maximum  
235 reduction on lipid content compared with control group (Figure 2A). This was confirmed by  
236 the quantitative Oil-Red-O staining measurements (Figure 3), which indicated that HWP  
237 grown in the presence of GPL exhibited a reduction of the lipid content by  $20.9\pm 4.0\%$   
238 ( $p<0.05$ ). In addition, IY and VIY reduced significantly ( $p<0.05$ ) the final lipid accumulation  
239 by  $10.4\pm 4.1\%$  and  $9.3\pm 2.5\%$ , respectively.

240 Analysis of the results obtained with KY, VY and LKP (Figure 3) showed no significant  
241 difference ( $p > 0.05$ ) when compared with control cells.

242 As we observed a reduction of the final lipid content after adipocytes incubation with GPL,  
243 IY, and VIY during the differentiation stage, we then examined if these peptides could  
244 potentially act on human adipose conversion at early or late stages of the differentiation  
245 programs.

246 For that purpose, 100  $\mu$ M of IY, GPL, and VIY were added to the differentiation medium or  
247 to the nutrition medium. The extent of differentiation process leading to the filling of  
248 adipocytes with lipid droplets was monitored by quantitative Oil-Red-O staining at day 16  
249 (Figure 4). When peptides were incubated with cells during the early stage of differentiation,  
250 GPL and IY reduced significantly ( $p < 0.05$ ) the final lipid accumulation by  $13.5 \pm 3.6\%$  and  
251  $8.3 \pm 0.3\%$ , respectively, while VIY had only a minor effect on the final lipid content (no  
252 significant  $p > 0.05$ ). However, none of the peptides tested affected significantly ( $p > 0.05$ ) the  
253 final lipid content when incubated during the second period of differentiation process.

### 254 ***3.3. Effect of marine cryptides on GPDH activity***

255 The activity of GPDH, involved in TG synthesis, was measured on day 8 after the addition of  
256 100  $\mu$ M of GPL, IY, VIY, KY, VY and LKP during differentiation. We measured a really low  
257 GPDH activity in undifferentiated pre-adipocytes (data not shown). As shown in Figure 5,  
258 GPDH activity decreased by  $33.7 \pm 10.6\%$  ( $p < 0.05$ ) in HWP cells incubated with GPL  
259 compared with untreated cells. Cells treated with IY also had a GPDH activity significantly  
260 ( $p < 0.05$ ) decreased by  $25.8 \pm 4.8\%$ . On the other hand, there was no significant ( $p > 0.05$ )  
261 change in the GPDH activity of HWP cells treated with other tested peptides (VY, VIY, KY  
262 or LKP).

263

#### 264 ***3.4. Effect of marine cryptides on the expression of adipogenic marker genes***

265 In order to assess appropriate genetic changes during the HWP cells differentiation, RT-PCR  
266 assay was first carried out for undifferentiated cells and cells after 8 days of differentiation,  
267 without peptide treatment. We checked that mRNA expression level of all genes tested  
268 increased as differentiation proceeded, supporting that the differentiation of pre-adipocyte was  
269 induced properly (data not shown).

270 We previously showed that GPL and IY decreased significantly the total lipid content and the  
271 GPDH activity indicating an action during early differentiation process. The effect of  
272 incubating HWP with GPL or IY was measured on the expression of markers of adipocyte  
273 differentiation (LPL, GLUT4, aP2 and AGT). In this study, we added the cryptide LKP as a  
274 negative control since no change on lipid content or GPDH activity was observed previously  
275 with this cryptide.

276 HWP were treated at day 8 with 100  $\mu$ M of the above mentioned peptides. The effect of GPL,  
277 IY and LKP on mRNA expression of adipogenic genes during HWP differentiation is  
278 presented Figure 6. The mRNA expression of GLUT4 and aP2 were significantly down-  
279 regulated by approximately two-fold ( $p<0.01$ ) after GPL treatment. When compared with  
280 untreated cells, the addition of GPL to the medium resulted also in a decrease of relative LPL  
281 and AGT mRNA expression to  $0.81\pm 0.09$  ( $p<0.05$ ) and  $0.70\pm 0.05$  ( $p<0.01$ ) respectively.

282 The mRNA expression level of LPL, GLUT4, aP2, and AGT were also down-regulated by IY  
283 treatment (Figure 6A, B, C, D) but in a lesser extent. However, LKP did not affect gene  
284 expression except for aP2 whose expression increased.

285 We then investigated whether, after 8 days of treatment during differentiation, GPL and IY  
286 could suppress adipogenesis through the PPAR $\gamma$  and C/EBP $\alpha$  pathways. GPL and IY

287 significantly down regulated mRNA expression of PPAR $\gamma$  ( $p<0.05$ ) (Figure 6E) and C/EBP $\alpha$   
288 ( $p<0.01$ ) (Figure 6F). However, the effect of IY were weaker than the one of GPL: the relative  
289 mRNA expression of PPAR $\gamma$  and C/EBP $\alpha$  were decreased to  $0.70\pm 0.09$  and  $0.47\pm 0.01$ ,  
290 respectively, compared to the control cells after GPL treatment (Figure 6E, F), while relative  
291 mRNA expression of PPAR $\gamma$  and C/EBP $\alpha$  were decreased to  $0.80\pm 0.09$  and to  $0.78\pm 0.06$   
292 when cells were incubated with IY.

#### 293 **4. Discussion**

294 In our study, we investigated *in vitro* the impact of bioactive peptides derived from marine  
295 organisms on the proliferation and the differentiation of human white pre-adipocytes cells in  
296 order to identify their possible uses in the control of human body mass. All the cryptides  
297 chosen in our investigation were reported to have an antihypertensive potential by the  
298 inhibition of ACE activity. The link between inhibitors of ACE and weight is currently under  
299 investigation and targeting the adipocyte cell cycle seems to be one of the solutions proposed  
300 against obesity.

301 We first defined the cryptide concentration of study and decided to work with a quite high  
302 concentration ( $100\mu\text{M}$ ) in order to discriminate quickly the most interesting peptides: those  
303 exerting an action on the development of adipocytes.

304 We demonstrated that all ACE inhibitory peptides could not affect the viability of HWP cells  
305 during proliferation and differentiation. Among 11 cryptides tested, 5 were found to be toxic  
306 for human adipocytes at a concentration of  $100\mu\text{M}$ : AKK, AP and VAP decreased  
307 preadipocytes viability while KW and VW exert their toxic effect during the differentiation  
308 stage. The decrease of cell numbers was probably due to the inhibition of cell division and/or  
309 induction of cell death by apoptosis in HWP. This was already demonstrated by a number of

310 natural products such as polyphenol compounds which were shown to inhibit preadipocytes  
311 proliferation and induce apoptosis. For example, quercetin and diphlorethohydroxycarmalol  
312 (DPHC) induced apoptosis in 3T3-L1 pre-adipocyte by decreasing mitochondria membrane  
313 potential, down-regulating poly (ADP-ribose) polymerase (PARP), Bcl-2 and activating  
314 caspase 3, Bax and Bak (Park et al., 2013; Rayalam et al., 2008). Kim et al. (2006) suggested  
315 that adipocytes deletion by apoptosis could be a contributor to body fat loss Thus, the use of  
316 AKK, AP and VAP might be an appropriate approach to address obesity through adipocyte  
317 deletion.

318 Our results also revealed that KW and VW exerted an antiproliferative effect on HWP during  
319 the differentiation period in contrast to the HWP in proliferation stage and more interestingly  
320 they decreased cell viability during early stage of differentiation. This action on the  
321 differentiation stage was already observed with an omega-3 fatty acid found in fish oil.  
322 Indeed, Kim et al. (2006) reported that docosahexaenoic acid (DHA) did not affect cell  
323 growth in 3T3-L1 pre-adipocytes. However, after initiation of differentiation, DHA treatment  
324 decreased cell viability with an increase in LDH release. They suggested that DHA treatment  
325 induced apoptosis in 3T3-L1 during mitotic clonal expansion, occurring during the first few  
326 days after initiation of differentiation. In fact, it was reported that two critical events occur  
327 during the early stage of differentiation: mitotic clonal expansion and an irreversible  
328 commitment to differentiation (Rayalam et al., 2008). We demonstrated that GPL, LKP, IY,  
329 KY and VIY have no effect on the viability of HWP neither during proliferation nor  
330 differentiation. So they did not target the adipocyte life cycle through controlling adipocyte  
331 hyperplasia. However, these peptides could act like botanical alkaloids on the commitment to  
332 differentiation. Indeed, berberine, which affects the viability of 3T3-L1 during proliferation  
333 and differentiation and the viability of HWP cells only during the differentiation, induced a



334 major inhibition of differentiation and finally suppressed adipocyte hypertrophy in both 3T3-  
335 L1 and HWP cells (Hu, Fahmy, Zjawiony, & Davies, 2010; Hu & Davies, 2009).

336 Therefore, the effect of GPL, IY, VIY, KY, VY and LKP on adipocyte differentiation was  
337 evaluated using Oil-Red-O staining, the measure of GPDH activity and the mRNA expression  
338 changes of adipocyte differentiation markers.

339 The Oil-Red-O staining demonstrated that GPL, IY and VIY significantly decreased final  
340 lipid content in HWP cells. This inhibition of lipid accumulation was associated with a  
341 decrease of GPDH activity, a marker of late adipocyte differentiation only for GPL and IY.

342 Since GPL and IY seem to affect at least partially adipocyte differentiation, we next  
343 investigated the expression of adipocyte specific genes at the same stage of cell cycle to  
344 determine their potential mechanism of action.

345 The adipocyte differentiation process is coordinated by two major classes of transcription  
346 factors, namely peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and CCAAT/enhancer  
347 binding proteins (C/EBPs) (Yim, Hosokawa, Mizushima, Yoshida, Saito, & Miyashita, 2011).  
348 These two key regulators of adipogenesis mediate the acquisition of adipocyte phenotype and  
349 act synergistically to promote adipogenesis (Rahman et al., 2008).

350 We measured in the current study the expression of LPL, GLUT4, aP2, AGT, PPAR $\gamma$ , and  
351 C/EBP $\alpha$ , genes in response to GPL and IY peptide treatment and observed a significant  
352 decrease of their expression levels during HWP cells differentiation between cryptide-treated  
353 groups and control-treated groups. Thus, the GPL and IY treatment of maturing HWP might  
354 inhibit at least partially adipogenic activity through the down regulation of PPAR $\gamma$  and  
355 C/EBP $\alpha$ .

356 Few previous studies reported some similar effect of peptides derived from other natural  
357 sources on adipocyte differentiation. For example, Kim et al. (2007) demonstrated that a  
358 tripeptide IQN, purified from a black soybean protein hydrolysate, inhibited adipogenesis of  
359 an adipose cell line derived from mouse embryo, through the inhibition of differentiation.  
360 More recently, Jung et al. (2011) showed that silk peptides were able to suppress the  
361 adipocyte differentiation in C3H10T1/2 and 3T3-L1 cell lines through inhibition of Notch  
362 pathway.

363 The sequences of our most active cryptides, GPL and IY, have been already identified as  
364 important in the regulation of various mechanisms. For example, the sequence GPL pertains  
365 to the glyproline family (Samonina, Ashmarin, & Lyapina, 2002), peptide family including  
366 proline containing linear peptides, whose most important effects are the suppression of some  
367 reactions of blood coagulation and platelet aggregation and the protection of gastric mucosa  
368 against various ulcerogenic factors. Moreover, the association of amino-acids from GPL and  
369 IY can be found in the sequence of insulin membrane receptor. GPLY forms a recognition  
370 signal for insulin receptor endocytosis (Berhanu, Anderson, Paynter & Wood, 1995). Thus,  
371 further work will be undertaken to measure the role of GPL and IY on the insulin pathway.

372

### 373 ***5. Conclusion***

374 Despite exhibiting high inhibitory activity of ACE (Ben Henda et al., 2013), LKP and VY  
375 have no effect on the proliferation and the differentiation of adipocyte cells, indicating that the  
376 ability of marine cryptides to affect adipogenesis is not correlated with their ACE-inhibitory  
377 activity.

378 We showed in this work that some marine peptides can act on adipose mass by targeting  
379 various stages in the life cycle of human white adipocytes. AP, VAP and AKK may induce  
380 inhibition of growth of preadipocytes during proliferation stage while KW and VW could  
381 decrease the number of preadipocytes engaged into the differentiation process. Treatment  
382 with GPL or IY inhibited adipogenesis through down-regulating adipocyte specific  
383 transcription factors.

384 It is important now to check lower concentration of peptides and better characterize the  
385 molecular way of action of GPL and IY peptides. We also wish to investigate the GPLY  
386 tetrapeptide effect on human white adipocyte cycle, in order to evaluate potent similarities  
387 and make a link between insulin pathway and GPL and IY cryptides.

388 These encouraging findings demonstrate for the first time that some cryptides, easily obtained  
389 from marine food products, can target the human white adipocyte life cycle. Thus, we believe  
390 that these marine cryptides have potential to become associated preventing/treating obesity  
391 agents.

392

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397

398

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472 **Table**

<b>Oligonucleotide</b>	<b>Sequences (5' to 3')</b>
Human PPAR $\gamma$ -R	GGCGGTCTCCACTGAGAATA
Human PPAR $\gamma$ -F	GAGCCCAAGTTTGAGTTTGC
Human C/EBP $\alpha$ -R	TTTAGCAGAGACGCGCACATTAC
Human C/EBP $\alpha$ -F	ATTGCCTAGGAACACGAAGCACGA
Human LPL-R	CTGGCATTGCAGGAAGTCTG
Human LPL-F	GCATCATCAGGAGAAAGACGA
Human $\alpha$ P2-R	TCTCTTTATGGTGGTTGATTTT
Human $\alpha$ P2-F	CAGTGTGAATGGGGATGTG
Human GLUT4-R	TTTCACCTCCTGCTCTAA
Human GLUT4-F	CGTCTTCCTTCTATTTGC
Human AGT-R	AGGCATAGTGAGGCTGGAT
Human AGT-F	CCTGGCTTTCAACACCTAC
Human $\beta$ -actin-R	GCCTCGTCGCCACATAG
Human $\beta$ -actin-F	GCCGTCTTCCCCTCCATC

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474 Table 1: Primer sequences used for Real time PCR

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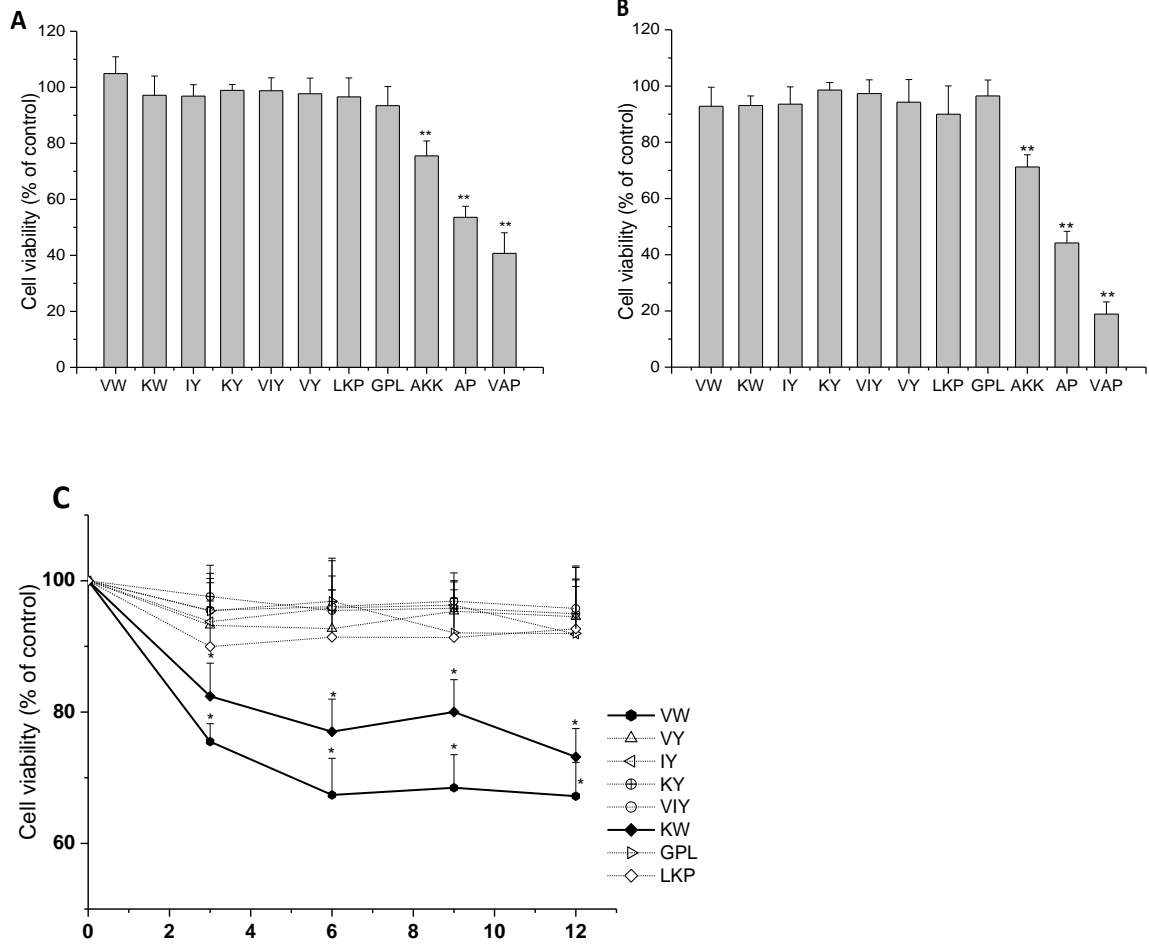
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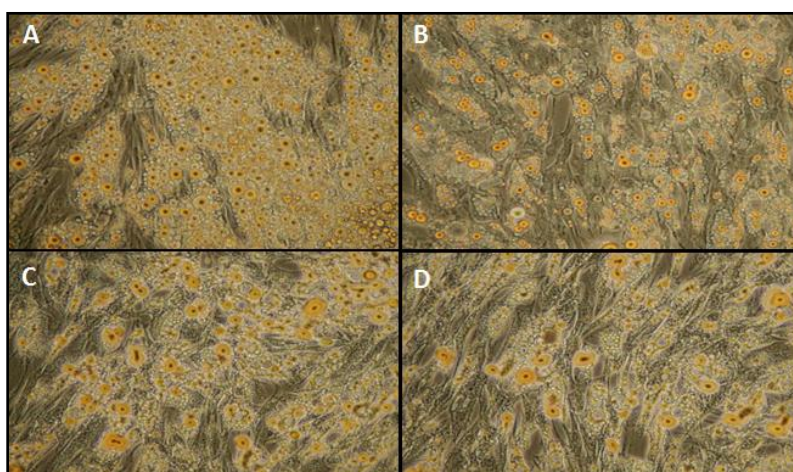
489 **Figures**



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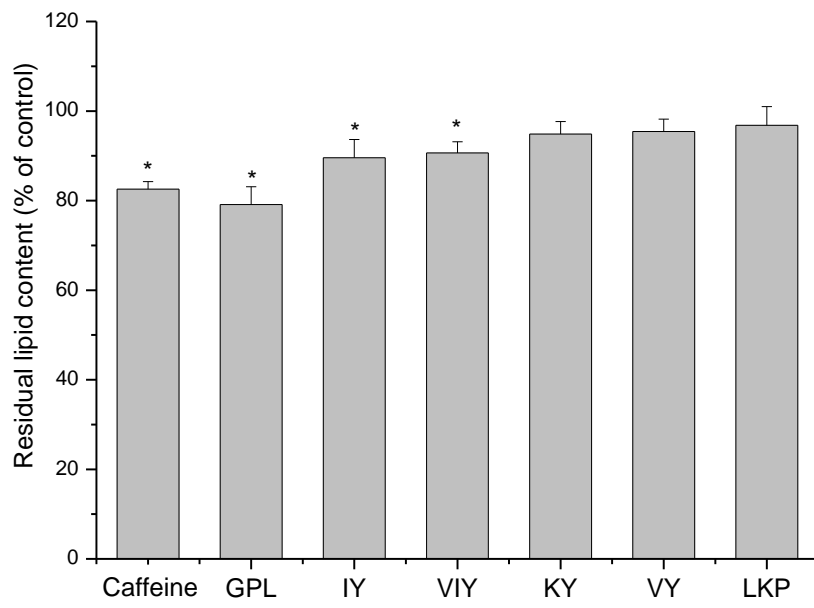
492 **Figure 1**



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495 **Figure 2**



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497 Figure 3

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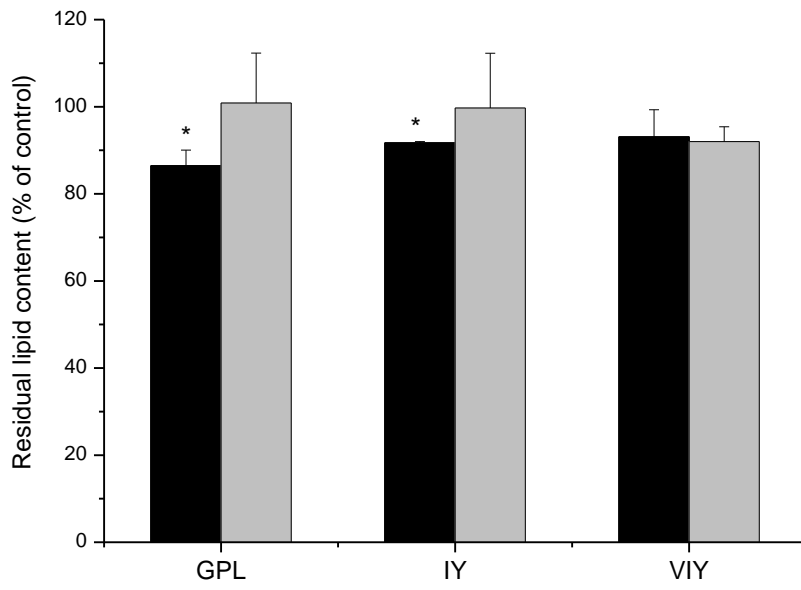
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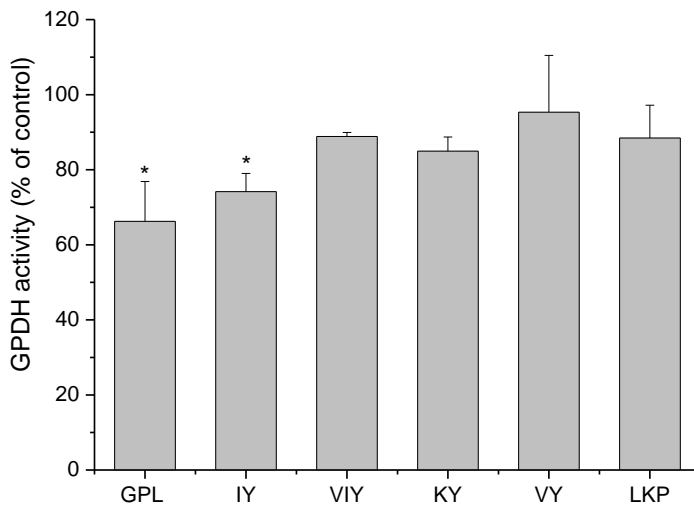
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505 Figure 4

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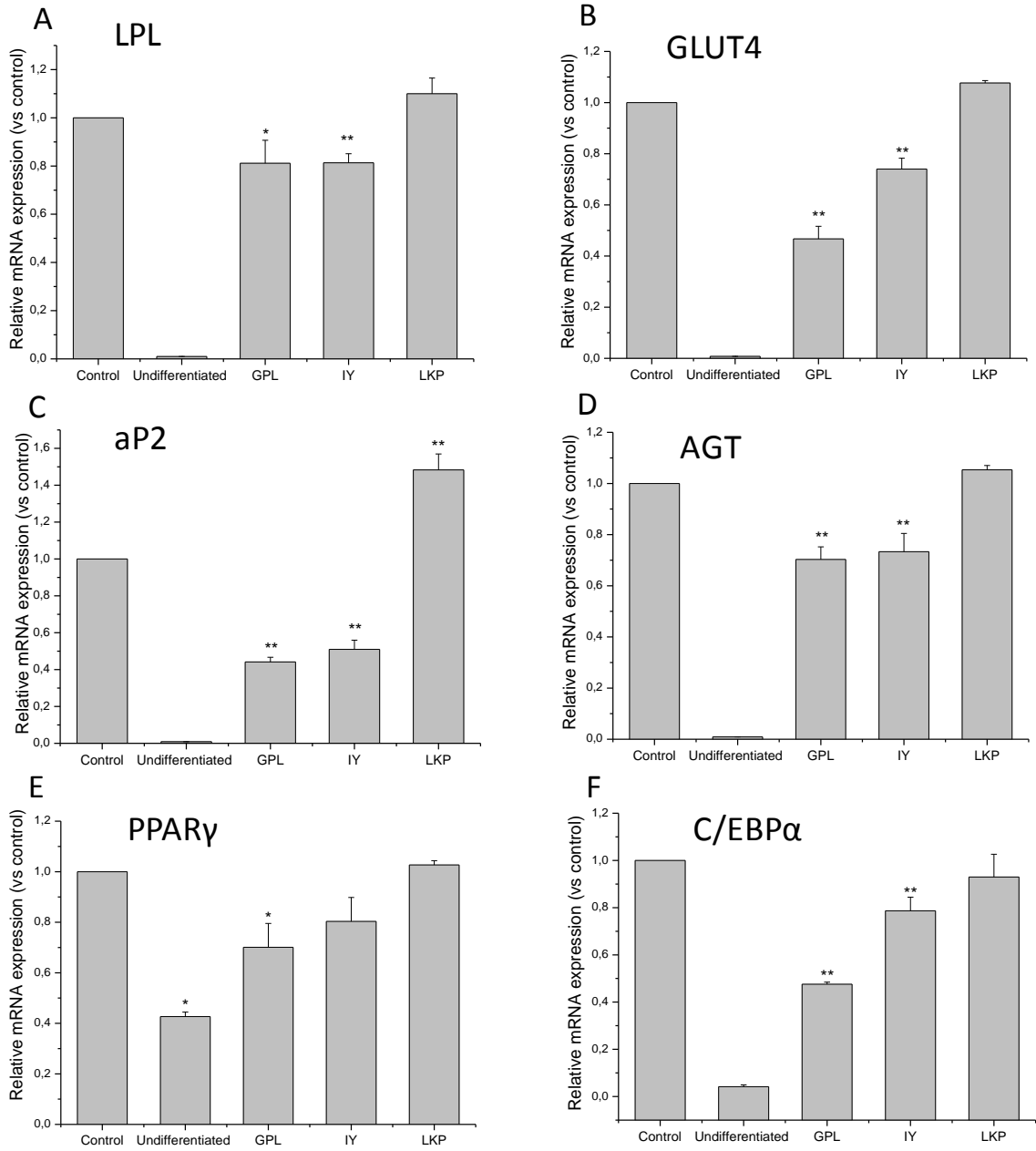
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515 Figure 5

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518 Figure 6

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520 FIGURE CAPTIONS:

521 Figure 1:

522 Effect of cryptides on cell viability of HWP during proliferation after 24 h (A) and 72 h (B) of  
523 treatment and after 3, 6, 9 and 12 days of differentiation (C). Cell viability was expressed as  
524 percentage of non-treated cells (control). (A) and (B) Pre-confluent cells were treated with  
525 100  $\mu$ M of peptides (VW, KW, IY, KY, VIY, VY, VIY, LKP, GPL, AKK, AP, VAP) at 24 h  
526 after cell plating. (C) Differentiation of HWP at confluence was induced and cells were  
527 treated with 100  $\mu$ M of peptides (VW, VY, IY, KY, VIY, KW, GPL, LKP) in appropriate  
528 medium. Values are means  $\pm$  SEM,  $n=24$  replicates. \*denotes significant difference compared  
529 with non-treated group ( $p < 0.05$ ) and. \*\* ( $p < 0.01$ ). Cryopreserved cells were obtained from a  
530 single donor.

531

532

533 Figure 2:

534 Effect of cryptides on lipid content of mature adipocytes. Cells were treated during  
535 differentiation period (from day 0 to day 16) control (A), 100  $\mu$ M of GPL (B), IY(C) and 100  
536  $\mu$ M caffeine (D).

537 Figure 3:

538 Effect of cryptides on residual lipid content of mature adipocytes. Values were presented as  
539 percentage of non-treated cells (control) optical density. Cells were treated throughout the  
540 differentiation period (from day 0 to day 16) with 100  $\mu$ M of caffeine, GPL, IY, VIY, KY,  
541 VY and LKP. Values are means  $\pm$  SEM,  $n=12$  replicates. \*denotes significant difference

542 compared with non-treated group ( $p < 0.05$ ). Cryopreserved cells were obtained from a single  
543 donor.

544

545 Figure 4:

546 Effect of cryptides on residual lipid content of mature adipocytes treated at different stage of  
547 the differentiation process. Values were expressed as percentage of non-treated cells (control).  
548 Cells were treated during differentiation process from day 0 to day 4 (■) or from day 4 to day  
549 16 (■) with 100  $\mu\text{M}$  of GPL, IY and VIY. Values are means  $\pm$  SEM,  $n=12$  replicates.  
550 \*denotes significant difference compared with non-treated group ( $p < 0.05$ ). Cryopreserved  
551 cells were obtained from a single donor.

552

553 Figure 5:

554 Effect of cryptides on Glycerol-3-phosphate dehydrogenase (GPDH) specific activity of  
555 HWP. GPDH activity was presented as percentage of the activity of non-treated cells  
556 (control). Cells were treated during differentiation from day 0 to day 8 in the presence of 100  
557  $\mu\text{M}$  of GPL, IY, VIY, KY, VY and LKP. Values are means  $\pm$  SEM,  $n=3$  replicates. \*denotes  
558 significant difference compared with non-treated group ( $p < 0.05$ ). Cryopreserved cells were  
559 obtained from a single donor.

560

561 Figure 6:

562 Effect of GPL, IY and LKP on mRNA expression of LPL (A), GLUT 4 (B), aP2 (C), AGT  
563 (D), PPAR $\gamma$  (E) and C/EBP $\alpha$  (F) during HWP differentiation. Cells were treated during

564 differentiation from day 0 to day 8 in the presence of 100  $\mu$ M of GPL, IY and LKP. The  
565 mRNA expression levels were measured by real time PCR and expressed as a ratio to non-  
566 treated cells (control) levels (=1.0) after normalization using the  $\beta$ -actin mRNA expression  
567 level. Values given are the means  $\pm$ S.D. (n=3), \*\* and \* denotes significant differences  
568 compared with non-treated cell (p<0.01) and (p<0.05). Cryopreserved cells were obtained  
569 from a single donor.