

1 **No effect of administration of unacylated ghrelin on**
2 **subcutaneous PC3 xenograft growth in a *Rag1*^{-/-} mouse**
3 **model of metabolic dysfunction**

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27 **Conflict of interest**

28 The authors declare no conflict of interest.

29

30 **Abstract**

31

32 Ghrelin is a peptide hormone which, when acylated, regulates appetite, energy
33 balance and a range of other biological processes. Ghrelin predominately circulates in its
34 unacylated form (unacylated ghrelin; UAG). UAG has a number of functions independent of
35 acylated ghrelin, including modulation of metabolic parameters and cancer progression. UAG
36 has also been postulated to antagonise some of the metabolic effects of acyl-ghrelin,
37 including its effects on glucose and insulin regulation. In this study, *Rag1*^{-/-} mice with high-
38 fat diet-induced obesity and hyperinsulinaemia were subcutaneously implanted with PC3
39 prostate cancer xenografts to investigate the effect of UAG treatment on metabolic
40 parameters and xenograft growth. Daily intraperitoneal injection of 100 µg/kg UAG had no
41 effect on xenograft tumour growth in mice fed normal rodent chow or 23% high-fat diet.
42 UAG significantly improved glucose tolerance in host *Rag1*^{-/-} mice on a high-fat diet, but did
43 not significantly improve other metabolic parameters. We hypothesise that UAG is not likely
44 to be an effective treatment for prostate cancer, with or without associated metabolic
45 syndrome.

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50 **Introduction**

51
52 The peptide hormone ghrelin is a circulating appetite-stimulating hormone which
53 regulates a number of other biological processes [1-3], including metabolism and energy
54 balance [1-4], and diseases such as cancer [5]. Ghrelin acts via its cognate receptor, the
55 growth hormone secretagogue receptor 1a (GHSR1a), a G protein-coupled receptor [6], and
56 one or more unknown alternative receptors [7-10]. In order to activate GHSR1a at
57 physiological concentrations, ghrelin must be acylated at its third residue, a serine [11, 12],
58 by the enzyme ghrelin *O*-acyl transferase (GOAT) [11, 12].

59 The major circulating form of ghrelin is its unmodified form, unacylated ghrelin
60 (UAG). UAG, which does not directly stimulate feeding [5], was initially considered to be
61 functionally inactive, but is now appreciated to bind to and activate a distinct, unknown
62 receptor [4, 13-18] and have a number of functions [19-22]. UAG plays roles in the
63 regulation of glucose and energy balance and has effects on cell proliferation [19-23].
64 Importantly, it may oppose some of the effects of acyl-ghrelin [16, 24-26] preventing the rise
65 in circulating glucose and insulin associated with acyl-ghrelin administration in rodents [22,
66 26, 27]. From these studies, it is apparent that UAG is an endocrine hormone in its own right
67 [20]. UAG and the truncated, cyclised UAG analogue AZP-531 prevented the development
68 of pre-diabetes in C57BL/6 mice fed a high-fat diet for two weeks, highlighting a potential of
69 unacylated forms of ghrelin as treatments for metabolic syndrome [27]. In human trials, UAG
70 had similar effects, improving glycaemic control and insulin sensitivity in patients with type
71 2 diabetes mellitus [28] and improving glucose handling and reducing free fatty acids in
72 healthy subjects when administered overnight as a continuous infusion [29]. AZP-531 also
73 had beneficial effects on glucose balance and led to weight loss in patients with type 2
74 diabetes mellitus in a phase I clinical trial [30]. Similar benefits have been observed in

75 patients with Prader-Willi syndrome, a genetic disorder associated with hyperghrelinæmia
76 and obesity [31].

77 Close to two decades of work has firmly established a role for the ghrelin axis in
78 cancer [5, 32-35]. This includes prostate cancer, a classical endocrine-related cancer and the
79 most commonly diagnosed cancer in American men after skin cancer [36], where acyl-
80 ghrelin increases cell proliferation and migration [5, 14, 37-46]. UAG also has functional
81 effects in several cancers, including prostate cancer [5, 32-34, 43]. In the PC3 prostate cancer
82 cell line, UAG has a biphasic effect, reducing cell proliferation at supraphysiological levels
83 (10nM-1µM) [14].

84 Studies investigating the role of UAG in prostate cancer have been limited to *in vitro*
85 experiments. *In vivo* studies are required, however, as obesity and overweight and co-

86 morbidities, including hyperinsulinaemia, are now recognised as critical risk factors for
87 numerous cancers [47-49]. These include cancer types with high-prevalence and mortality,
88 such as tumours of the prostate, endometrium, breast, and gastrointestinal system [47-55].

89 Obesity and increased body mass have been associated with increased risk of advanced
90 prostate cancer, more aggressive and high-grade disease and increased risk of death from
91 prostate cancer [56-59]. Castration-resistant prostate cancer (CRPC) occurs when prostate
92 cancer recurs after remission from androgen-targeted therapies (ATT) [60]. Treatments for
93 CRPC are limited and this stage of the disease often results in the formation of painful,
94 metastatic bone lesions and associated morbidity and mortality [61-63]. Metabolic syndrome
95 and hyperinsulinaemia are common side effects of ATT [64, 65] and may also further
96 accelerate the progression to CRPC [48, 58, 66-68]. As UAG reduces prostate cancer
97 proliferation *in vitro* [14] and has potential beneficial metabolic effects *in vivo*, we examined
98 the effect of UAG in our model of metabolic dysfunction: *Rag1*^{-/-} mice fed a high-fat diet,
99 with subcutaneous prostate cancer cell line xenografts [69].

100

101 **Materials and Methods**

102 **Cell Culture**

103 Human prostate cancer cell lines were obtained from the American Type Culture
104 Collection (ATCC, Manassas, VA, USA). The PC3 prostate cancer cell line was cultured in
105 Roswell Park Memorial Institute 1640 medium (RPMI-1640) and supplemented with 10%
106 (v/v) Fetal Calf serum (FCS) (Thermo Fisher Scientific, Waltham, MA, USA), 50 units/ml
107 penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific). Cells were tested negative
108 for *Mycoplasma*.

109

110 **Hyperinsulinaemic *Rag1*^{-/-} mouse model treated with unacylated
111 ghrelin (UAG)**

112 To determine the metabolic effect of UAG in an engraftable mouse model of
113 hyperinsulinaemia [69], male recombination-activation gene deficient mice (B6.SVJ129-
114 *Rag1*^{tm1Bal}/Arc; *Rag1*^{-/-}) (Jackson Laboratories, supplied by Animal Resource Centre,
115 Murdoch, WA, Australia) were weaned onto a diet of low-fat normal chow (LFD) or a
116 Western-style, high-fat diet (HFD; 23% fat, SF04-027, Specialty Feeds, WA) [69]. After two
117 weeks on the diet, mice were subcutaneously injected into the left flank with 1×10⁶ PC3 cells
118 diluted 1:1 in growth factor reduced, phenol red-free Matrigel (Corning, NY, USA). Tumours
119 were allowed to grow until a volume of approximately 50-100 mm³ was reached, when mice
120 were randomly divided into two experimental groups. Mice then received daily
121 intraperitoneal injections of 100 µg/kg UAG (Mimotopes, Mulgrave, Vic, Australia) (n=6
122 HFD, n=10 LFD) (a dose previously determined to inhibit breast cancer growth *in vivo* [8])
123 or phosphate buffered saline (PBS) control (n=8 HFD, n=10 LFD) for 16 days. Tumour

124 volume was calculated by measuring subcutaneous tumour length and width twice weekly
125 using digital calipers (ProSciTech, Kirwan, QLD, Australia). Tumour volume was calculated
126 using the equation ‘tumour volume = (width \times length²)/2’ [70]. Bodyweight was measured
127 twice weekly. At endpoint, tumours and adipose tissue (epididymal fat pad and interscapular
128 brown adipose tissue) were excised and weighed. Fasting blood glucose was measured at
129 endpoint and blood was collected by cardiac puncture for serum biochemical measurements.
130 Surrogate indices of insulin resistance, insulin sensitivity, and steady state β -cell function
131 were determined using the homeostatic model for assessment calculator (HOMA2), available
132 from the Oxford Centre for Diabetes, Endocrinology and Metabolism [71], using measured
133 fasting glucose and insulin levels.

134

135 **Statistics**

136 Statistical analyses were performed using GraphPad Prism v.6.01 software (GraphPad
137 Software, Inc., San Diego, CA). Kruskal-Wallis (three or more groups) and Mann-Whitney
138 *U*-test (two groups) tests used for non-normally distributed data, while a two-way ANOVA
139 with Tukey’s post-hoc test used for normally distributed data. $P \leq 0.05$ was considered to be
140 statistically significant.

141 **Results**

142 **No effect of intraperitoneal administration of UAG on PC3 143 xenograft growth in obese, hyperinsulinaemic *Rag1*^{-/-} mice**

144 No significant differences in tumour volume over the treatment period or tumour
145 weight ($P=0.57$) and volume at endpoint ($P=0.55$) were observed between UAG-treated and
146 untreated obese mice (14 days of treatment) (Mann-Whitney test, Fig. 1A-C). An increase in
147 insulin sensitivity ($P=0.70$) and reduction in body weight ($P=0.08$), epididymal fat pad

148 weight ($P=0.26$), interscapular brown adipose tissue weight ($P=0.12$), fasting blood glucose
149 ($P=0.50$), fasting blood insulin ($P=0.90$), insulin resistance ($P=0.70$), and steady-state β -cell
150 function ($P=0.22$) was observed in the UAG treatment group in HFD-fed $Rag1^{-/-}$ mice –
151 however, these changes were not statistically significant (Fig. 1D-L). There was a significant
152 difference in blood glucose at 30 minutes following glucose challenge in HFD-fed UAG-
153 treated mice compared to PBS controls at endpoint (after 16 days of treatment) ($21.6 \pm$
154 1.2mM , $n=6$ vs $25.4 \pm 1.9\text{mM}$, $n=5$, $P=0.02$, two-way ANOVA with post-hoc test, Fig. 1G),
155 however, this was not observed at other time points, suggesting no major change in glucose
156 tolerance. There was no significant difference in fasting blood glucose ($P=0.50$), blood
157 insulin concentration ($P = 0.90$, Mann-Whitney test, Fig. 1I), insulin resistance ($P = 0.70$,
158 Mann-Whitney test, Fig. 1J), or insulin sensitivity ($P=0.70$, Mann-Whitney test, Fig. 1L).

159

160 **Fig. 1. Unacylated ghrelin (UAG) affects glucose tolerance but has no effect on tumour**
161 **volume or on other metabolic parameters.** $Rag1^{-/-}$ mice fed a 23% high-fat diet (HFD) or
162 low-fat diet (LFD) were injected with subcutaneous PC3 xenografts and administered UAG
163 (100 $\mu\text{g}/\text{kg}/\text{day}$, i.p.) ($n=6$ HFD, $n=10$ LFD) or PBS control ($n=8$ HFD, $n=10$ LFD) once
164 tumours were palpable. Mean \pm s.e.m. * $P \leq 0.05$. (A) Tumour volume (mm^3) measured over
165 time ($P=0.57$), (B) tumour volume (mm^3) ($P=0.55$) and (C) tumour weight (g) measured at
166 experimental endpoint were not significantly different between UAG- and PBS-treated mice
167 fed HFD or LFD. Mean \pm s.e.m. Mann-Whitney test. (D) Body weight (g) of mice at
168 endpoint ($P=0.08$), (E) epididymal fat pad weight (g) ($P=0.26$) and (F) interscapular brown
169 adipose tissue weight (g) ($P=0.12$) were not significantly different in UAG-treated mice
170 compared to PBS-treated mice. Mean \pm s.e.m. Mann-Whitney test. (G) HFD-fed UAG-
171 treated mice ($n=6$) had significantly lower blood glucose 30 min post-glucose challenge
172 compared to HFD-fed PBS treated mice ($n=8$), determined by intraperitoneal glucose

173 tolerance test (IPGTT). Mean \pm s.e.m. Two-way ANOVA. * $P=0.025$. (H) Fasting blood
174 glucose (mM) ($P=0.50$) and (I) fasting blood insulin (ng/ml) were not altered in UAG-treated
175 compared to PBS-treated mice on either diet. Mean \pm s.e.m. $P=0.90$. Mann-Whitney test. (J)
176 Insulin resistance (HOMA-IR) ($P=0.70$), (K) steady state β -cell function (HOMA% B)
177 ($P=0.22$) and (L) insulin sensitivity (HOMA% S) ($P=0.70$) were not altered in UAG-treated
178 compared to PBS-treated mice on either diet. Mean \pm s.e.m. Mann-Whitney test.

179 **Discussion**

180
181 It has recently been recognised that UAG can under some conditions act as a
182 functional ghrelin inhibitor, reducing ghrelin-mediated increases in plasma glucose [22, 26,
183 28, 72] and lipid [27, 29]. As the ghrelin axis also plays a role in the progression of a number
184 of endocrine-related cancers [5, 32-34], including prostate cancer [5, 43], we hypothesised
185 that UAG may have beneficial effects in advanced prostate cancer associated with metabolic
186 syndrome. To evaluate this hypothesis, we examined the effect of UAG on a prostate cancer
187 cell line *in vivo*.

188 In our diet-induced hyperinsulinaemic *Rag1*^{-/-} mouse model [69], we investigated the
189 effect of supraphysiological systemic UAG treatment (100 μ g/kg/day) on metabolic
190 parameters and PC3 prostate cancer xenograft growth. No differences in metabolic
191 parameters (fasting blood glucose, fasting blood insulin, insulin resistance, steady-state β -cell
192 function, and insulin sensitivity) were observed with UAG treatment in HFD-fed mice. Other
193 studies have found that UAG prevents insulin resistance and hyperglycaemia in short-term
194 HFD-fed mice [73], observations which may stem from the ability of UAG to cross the
195 blood-brain barrier and oppose the central actions of ghrelin on energy homeostasis [74].
196 Furthermore, in human clinical trials UAG improved glucose and lipid metabolism in healthy
197 [29] and diabetic patients [28]. In our study, a decrease in bodyweight, epididymal fat pad

198 weight, and interscapular brown adipose tissue was observed in HFD-fed UAG treated mice
199 but this difference was not statistically significant. UAG did significantly reduce blood
200 glucose levels at 30 minutes post-glucose challenge in HFD, but not LFD-fed mice, however.
201 This is similar to other studies, which have only found positive effects of UAG on glucose
202 tolerance in obese patients [72]. Similarly, in clinical trials, AZP-531 (a cyclised, truncated
203 analogue of UAG) improved food-related behaviour, waist circumference, and glucose
204 tolerance in Prader-Willi syndrome patients, but had no effect on body weight [31]. AZP-531
205 also prevents HFD-induced weight gain, insulin resistance, and impairment of glucose
206 tolerance in mice [27].

207 To the best of our knowledge, this is the first report on the effects of UAG on cancer
208 cell line xenograft growth *in vivo*. While our study and others show somewhat promising
209 effects of UAG treatment on metabolic parameters, systemic UAG administration had no
210 effect on prostate tumour xenograft size in mice fed a low-fat or high-fat diet. While
211 preliminary, our study suggests that UAG administration, or targeting of endocrine UAG,
212 may have limited therapeutic potential for prostate cancer, in patients with and without
213 symptoms of metabolic syndrome.

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215
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225

226 **References**

227

228 1. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin
229 enhances appetite and increases food intake in humans. *J. Clin Endocrinol. Metab.*
230 2001;86(12): 5992. doi: 10.1210/jcem.86.12.8111.

231 2. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature.*
232 2000;407(6806): 908-13. doi: 10.1038/35038090.

233 3. Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, et al. Post-
234 prandial decrease of circulating human ghrelin levels. *J. Endocrinol. Invest.* 2001;24(6):
235 RC19-21.

236 4. Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, et al.
237 Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced
238 hypoglycemia, and leptin administration. *Biochem. Biophys. Res. Commun.*
239 2001;281(5): 1220-5. doi: 10.1006/bbrc.2001.4518.

240 5. Chopin LK, Seim I, Walpole CM, Herington AC. The ghrelin axis--does it have an
241 appetite for cancer progression? *Endocrine Rev.* 2012;33(6): 849 - 91.

242 6. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-
243 hormone-releasing acylated peptide from stomach. *Nature.* 1999;402(6762): 656-60. doi:
244 10.1038/45230.

245 7. Chopin L, Walpole C, Seim I, Cunningham P, Murray R, Whiteside E, et al. Ghrelin and
246 cancer. *Mol. Cell. Endocrinol.* 2011;340(1): 65-9. doi: 10.1016/j.mce.2011.04.013.

247 8. CheukMan Cherie A, Kara LB, Maria MD, Brid C, Jason EC, John BF, et al. Des-acyl
248 ghrelin suppresses breast cancer cell growth *in vitro* and *in vivo*. Meeting Abstracts:
249 Endocrine Society; 2016. p. FRI-065.

250 9. Docanto MM, Yang F, Callaghan B, Au CC, Ragavan R, Wang X, et al. Ghrelin and des-
251 acyl ghrelin inhibit aromatase expression and activity in human adipose stromal cells:
252 suppression of cAMP as a possible mechanism. Breast Cancer Res. Treat. 2014;147(1):
253 193-201. doi: 10.1007/s10549-014-3060-1.

254 10. Callaghan B, Furness JB. Novel and conventional receptors for ghrelin, desacyl-ghrelin,
255 and pharmacologically related compounds. Pharmacol. Rev. 2014;66(4):984-1001. doi:
256 10.1124/pr.113.008433.

257 11. Yang J, Zhao TJ, Goldstein JL, Brown MS. Inhibition of ghrelin O-acyltransferase
258 (GOAT) by octanoylated pentapeptides. Proc. Natl. Acad. Sci. USA 2008;105(31):
259 10750-5. doi: 10.1073/pnas.0805353105.

260 12. Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, et al.
261 Ghrelin octanoylation mediated by an orphan lipid transferase. Proc. Natl. Acad. Sci.
262 USA 2008;105(17): 6320-5. doi: 10.1073/pnas.0800708105.

263 13. Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoi S, Fubini A, et al. Ghrelin
264 and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through
265 ERK1/2 and PI 3-kinase/AKT. J. Cell Biol. 2002;159(6):1029-37. doi:
266 10.1083/jcb.200207165.

267 14. Cassoni P, Ghe C, Marrocco T, Tarabria E, Allia E, Catapano F, et al. Expression of
268 ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in
269 human prostate neoplasms and related cell lines. Eur. J. Endocrinol. 2004;150(2): 173-
270 84.

271 15. Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, et al. Identification,
272 characterization, and biological activity of specific receptors for natural (ghrelin) and
273 synthetic growth hormone secretagogues and analogs in human breast carcinomas and
274 cell lines. *J. Clin. Endocrinol. Metab.* 2001;86(4): 1738-45.

275 16. Gauna C, Delhanty PJD, Hofland LJ, Janssen JAMJL, Broglio F, Ross RJM, et al.
276 Ghrelin Stimulates, Whereas Des-Octanoyl Ghrelin Inhibits, Glucose Output by Primary
277 Hepatocytes. *J. Clin. Endocrinol. Metab.* 2005;90(2): 1055-60.

278 17. Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, et al. Des-acyl
279 ghrelin induces food intake by a mechanism independent of the growth hormone
280 secretagogue receptor. *Endocrinology.* 2006;147(5): 2306-14. doi: 10.1210/en.2005-
281 1357.

282 18. Filigheddu N, Gnocchi VF, Coscia M, Cappelli M, Porporato PE, Taulli R, et al. Ghrelin
283 and des-acyl ghrelin promote differentiation and fusion of C2C12 skeletal muscle cells.
284 *Mol. Biol. Cell* 2007;18(3): 986-94. doi: 10.1091/mbc.E06-05-0402.

285 19. Delhanty PJ, van der Eerden BC, van der Velde M, Gauna C, Pols HA, Jahr H, et al.
286 Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated
287 protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of
288 GHS-R1a. *J. Endocrinol.* 2006;188(1): 37-47. doi: 10.1677/joe.1.06404.

289 20. Delhanty PJ, Sun Y, Visser JA, van Kerkwijk A, Huisman M, van Ijcken WF, et al.
290 Unacylated ghrelin rapidly modulates lipogenic and insulin signaling pathway gene
291 expression in metabolically active tissues of GHSR deleted mice. *PLoS one.* 2010;5(7):
292 e11749. doi: 10.1371/journal.pone.0011749.

293 21. Baragli A, Ghe C, Arnoletti E, Granata R, Ghigo E, Muccioli G. Acylated and
294 unacylated ghrelin attenuate isoproterenol-induced lipolysis in isolated rat visceral

295 adipocytes through activation of phosphoinositide 3-kinase gamma and
296 phosphodiesterase 3B. *Biochim. Biophys. Acta.* 2011;1811(6): 386-96.

297 22. Broglio F, Gottero C, Prodam F, Gauna C, Muccioli G, Papotti M, et al. Non-acylated
298 ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin
299 in humans. *J. Clin. Endocrinol. Metab.* 2004;89(6): 3062-5. doi: 10.1210/jc.2003-
300 031964.

301 23. Au CC, Docanto MM, Zahid H, Raffaelli FM, Ferrero RL, Furness JB, et al. Des-acyl
302 ghrelin inhibits the capacity of macrophages to stimulate the expression of aromatase in
303 breast adipose stromal cells. *J. Steroid Biochem. Mol. Biol.* 2017;170: 49-53. doi:
304 10.1016/j.jsbmb.2016.07.005.

305 24. Kumar R, Salehi A, Rehfeld JF, Höglund P, Lindström E, Håkanson R. Proghrelin
306 peptides: Desacyl ghrelin is a powerful inhibitor of acylated ghrelin, likely to impair
307 physiological effects of acyl ghrelin but not of obestatin: A study of pancreatic
308 polypeptide secretion from mouse islets. *Reg. Peptides.* 2010;164(2-3): 65-70. doi:
309 <http://doi.org/10.1016/j.regpep.2010.06.005>.

310 25. Neary NM, Druce MR, Small CJ, Bloom SR. Acylated ghrelin stimulates food intake in
311 the fed and fasted states but desacylated ghrelin has no effect. *Gut.* 2006;55(1): 135.

312 26. Gauna C, Meyler FM, Janssen JAMJL, Delhanty PJD, Abribat T, van Koetsveld P, et al.
313 Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination
314 of acylated plus unacylated ghrelin strongly improves insulin sensitivity. *J. Clin.*
315 *Endocrinol. Metab.* 2004;89(10): 5035-42. doi: 10.1210/jc.2004-0363.

316 27. Delhanty PJ, Huisman M, Baldeon-Rojas LY, van den Berge I, Grefhorst A, Abribat T,
317 et al. Des-acyl ghrelin analogs prevent high-fat-diet-induced dysregulation of glucose
318 homeostasis. *FASEB journal : official publication of the Federation of American*

319 Societies for Experimental Biology. 2013;27(4):1690-700. Epub 2013/01/10. doi:
320 10.1096/fj.12-221143. PubMed PMID: 23299855.

321 28. Ozcan B, Neggers SJ, Miller AR, Yang HC, Lucaites V, Abribat T, et al. Does des-acyl
322 ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated
323 ghrelin levels? European journal of endocrinology / European Federation of Endocrine
324 Societies. 2014;170(6):799-807. Epub 2013/07/19. doi: 10.1530/eje-13-0347. PubMed
325 PMID: 23864339.

326 29. Benso A S-PD, Prodam F, Gramaglia E, Granata R, van der Lely AJ, Ghigo E, Broglio
327 F. Metabolic effects of overnight continuous infusion of unacylated ghrelin in humans.
328 Eur J Endocrinol 2012;166(5): 911.

329 30. Allas S, Delale T, Ngo N, Julien M, Sahakian P, Ritter J, et al. Safety, tolerability,
330 pharmacokinetics and pharmacodynamics of AZP-531, a first-in-class analogue of
331 unacylated ghrelin, in healthy and overweight/obese subjects and subjects with type 2
332 diabetes. Diabetes, Obesity Metab. 2016;18(9): 868-74. doi: 10.1111/dom.12675.

333 31. Allas S, Caixàs A, Poitou C, Coupaye M, Thuilleaux D, Lorenzini F, et al. AZP-531, an
334 unacylated ghrelin analog, improves food-related behavior in patients with Prader-Willi
335 syndrome: A randomized placebo-controlled trial. PloS one. 2018;13(1): e0190849. doi:
336 10.1371/journal.pone.0190849.

337 32. Fung JNT, Jeffery PL, Lee JD, Seim I, Roche D, Obermair A, et al. Silencing of ghrelin
338 receptor expression inhibits endometrial cancer cell growth in vitro and in vivo. Am. J.
339 Physiol. 2013;305(2): E305-13.

340 33. Lin TC, Hsiao M. Ghrelin and cancer progression. Biochim. Biophys. Acta.
341 2017;1868(1): 51-7. doi: 10.1016/j.bbcan.2017.02.002.

342 34. Grönberg M, Ahlin C, Naeser Y, Janson ET, Holmberg L, Fjällskog M-L. Ghrelin is a
343 prognostic marker and a potential therapeutic target in breast cancer. *PloS one*.
344 2017;12(4):e 0176059. doi: 10.1371/journal.pone.0176059.

345 35. Zhu J, Yao J, Huang R, Wang Y, Jia M, Huang Y. Ghrelin promotes human non-small
346 cell lung cancer A549 cell proliferation through PI3K/Akt/mTOR/P70S6K and ERK
347 signaling pathways. *Biochem. Biophys. Res. Commun.* 2018;498(3): 616-20. doi:
348 <https://doi.org/10.1016/j.bbrc.2018.03.031>.

349 36. American Cancer Society. *Cancer Facts & Figures 2018*. Atlanta: American Cancer
350 Society; 2018.

351 37. Bertaccini A, Pernetti R, Marchiori D, Pagotto U, Palladoro F, Palmieri F, et al.
352 Variations in blood ghrelin levels in prostate cancer patients submitted to hormone
353 suppressive treatment. *Anticancer Res.* 2009;29(4): 1345-8.

354 38. Mungan NA, Eminferzane S, Mungan AG, Yesilli C, Seckiner I, Can M, et al.
355 Diagnostic value of serum ghrelin levels in prostate cancer. *Urologia internationalis*.
356 2008;80(3):245-8. doi: 10.1159/000127334.

357 39. Malendowicz W, Ziolkowska A, Szyszka M, Kwias Z. Elevated blood active ghrelin and
358 unaltered total ghrelin and obestatin concentrations in prostate carcinoma. *Urologia
359 internationalis*. 2009;83(4): 471-5. doi: 10.1159/000251190.

360 40. Jeffery PL, Herington AC, Chopin LK. Expression and action of the growth hormone
361 releasing peptide ghrelin and its receptor in prostate cancer cell lines. *J. Endocrinol.*
362 2002;172(3): R7-R11.

363 41. Yeh AH, Jeffery PL, Duncan RP, Herington AC, Chopin LK. Ghrelin and a novel
364 preproghrelin isoform are highly expressed in prostate cancer and ghrelin activates
365 mitogen-activated protein kinase in prostate cancer. *Clin. Cancer Res.* 2005;11(23):
366 8295-303.

367 42. Lanfranco F, Baldi M, Cassoni P, Bosco M, Ghe C, Muccioli G. Ghrelin and prostate
368 cancer. *Vitamins Hormones*. 2008;77: 301-24. doi: 10.1016/s0083-6729(06)77013-3.

369 43. Seim I, Jeffery PL, de Amorim L, Walpole CM, Fung J, Whiteside EJ, et al. Ghrelin O-
370 acyltransferase (GOAT) is expressed in prostate cancer tissues and cell lines and
371 expression is differentially regulated in vitro by ghrelin. *Repro. Biol. Endocrinol.*
372 2013;11(1): 70.

373 44. Duxbury MS, Waseem T, Ito H, Robinson MK, Zinner MJ, Ashley SW, et al. Ghrelin
374 promotes pancreatic adenocarcinoma cellular proliferation and invasiveness. *Biochem.*
375 *Biophys. Res. Commun.* 2003;309(2): 464-8.

376 45. Waseem T, Duxbury M, Ito H, Ashley SW, Robinson MK. Exogenous ghrelin modulates
377 release of pro-inflammatory and anti-inflammatory cytokines in LPS-stimulated
378 macrophages through distinct signaling pathways. *Surgery*. 2008;143(3): 334-42. doi:
379 10.1016/j.surg.2007.09.039.

380 46. Dixit VD, Weeraratna AT, Yang H, Bertak D, Cooper-Jenkins A, Riggins GJ, et al.
381 Ghrelin and the growth hormone secretagogue receptor constitute a novel autocrine
382 pathway in astrocytoma motility. *J. Biol. Chem.* 2006;281(24): 16681-90. doi:
383 10.1074/jbc.M600223200.

384 47. Hammarsten J, Hogstedt B. Hyperinsulinaemia: a prospective risk factor for lethal
385 clinical prostate cancer. *Eur. J. Cancer* 2005;41(18): 2887-95. doi:
386 10.1016/j.ejca.2005.09.003.

387 48. Ma J, Li H, Giovannucci E, Mucci L, Qiu W, Nguyen PL, et al. Prediagnostic body-mass
388 index, plasma C-peptide concentration, and prostate cancer-specific mortality in men
389 with prostate cancer: a long-term survival analysis. *The Lancet Oncology*. 2008;9(11):
390 1039-47. doi: 10.1016/s1470-2045(08)70235-3.

391 49. Brooke Steele, Cheryll C. Thomas, S. Jane Henley, Greta M. Massetti, Deborah A.
392 Galuska, Tanya Agurs-Collins, et al. Vital Signs: Trends in Incidence of Cancers
393 Associated with Overweight and Obesity — United States, 2005–2014 U.S. Department
394 of Health and Human Services, Centers for Disease Control and Prevention, 2017.
395 MMWR Morb. Mort. Wkly Rep. 2017; 66(39):1052-1058.

396 50. Souaze F, Dupouy S, Viardot-Foucault V, Bruyneel E, Attoub S, Gespach C, et al.
397 Expression of neurotensin and NT1 receptor in human breast cancer: a potential role in
398 tumor progression. Cancer Res. 2006;66(12): 6243-9. doi: 10.1158/0008-5472.can-06-
399 0450.

400 51. Zhang Y, Zhu S, Yi L, Liu Y, Cui H. Neurotensin receptor1 antagonist SR48692 reduces
401 proliferation by inducing apoptosis and cell cycle arrest in melanoma cells. Mol. Cell.
402 Biochem. 2014;389(1-2): 1-8. doi: 10.1007/s11010-013-1920-3.

403 52. Brown M, Vale W. Effects of neurotensin and substance P on plasma insulin, glucagon
404 and glucose levels. Endocrinology. 1976;98(3): 819-22. doi: 10.1210/endo-98-3-819.

405 53. Sehgal I, Powers S, Huntley B, Powis G, Pittelkow M, Maihle NJ. Neurotensin is an
406 autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer.
407 Proc. Natl. Acad. Sci. USA. 1994;91(11): 4673-7. Epub 1994/05/24.

408 54. Vias M, Burtt G, Culig Z, Veerakumarasivam A, Neal DE, Mills IG. A role for
409 neurotensin in bicalutamide resistant prostate cancer cells. The Prostate. 2007;67(2):
410 190-202. doi: 10.1002/pros.20518.

411 55. Vidal Samuel J, Rodriguez-Bravo V, Quinn SA, Rodriguez-Barrueco R, Lujambio A,
412 Williams E, et al. A targetable GATA2-IGF2 axis confers aggressiveness in lethal
413 prostate cancer. Cancer Cell. 2015;27(2): 223-39. doi:
414 <http://dx.doi.org/10.1016/j.ccr.2014.11.013>.

415 56. Perez-Cornago A, Key TJ, Allen NE, Fensom GK, Bradbury KE, Martin RM, et al.
416 Prospective investigation of risk factors for prostate cancer in the UK Biobank cohort
417 study. *British J. Cancer.* 2017;117: 1562. doi: 10.1038/bjc.2017.312

418 57. Discacciati A, Orsini N, Wolk A. Body mass index and incidence of localized and
419 advanced prostate cancer--a dose-response meta-analysis of prospective studies. *Annals*
420 *Oncol.* 2012;23(7):1665-71. doi: 10.1093/annonc/mdr603.

421 58. Gunter JH, Sarkar PL, Lubik AA, Nelson CC. New players for advanced prostate cancer
422 and the rationalisation of insulin-sensitising medication. *International journal of cell*
423 *biology.* 2013;2013: 834684. doi: 10.1155/2013/834684.

424 59. World Cancer Research Fund International/American Institute for Cancer Research
425 Continuous Update Project Report: Diet, Nutrition, Physical Activity, and Prostate
426 Cancer. 2014. Available at: www.wcrf.org/sites/default/files/Prostate-Cancer-2014-Report.pdf

428 60. Grayhack JT, Keeler TC, Kozlowski JM. Carcinoma of the prostate. Hormonal therapy.
429 *Cancer.* 1987;60(3 Suppl): 589-601.

430 61. Vela I, Gregory LS, Gardiner EM, Clements JA, Nicol DL. Bone and prostate cancer cell
431 interactions in metastatic prostate cancer. *BJU International.* 2007;99(4): 735-42.

432 62. Loberg RD, Gayed BA, Olson KB, Pienta KJ. A paradigm for the treatment of prostate
433 cancer bone metastases based on an understanding of tumor cell-microenvironment
434 interactions. *J. Cell Biol.* 2005;96(3): 439-46. doi: 10.1002/jcb.20522.

435 63. Sturge J, Caley MP, Waxman J. Bone metastasis in prostate cancer: emerging therapeutic
436 strategies. *Nature Rev. Clin. Oncol.* 2011;8:357-68. doi: 10.1038/nrclinonc.2011.67.

437 64. Senmaru T, Fukui M, Okada H, Mineoka Y, Yamazaki M, Tsujikawa M, et al.
438 Testosterone deficiency induces markedly decreased serum triglycerides, increased small
439 dense LDL, and hepatic steatosis mediated by dysregulation of lipid assembly and

440 secretion in mice fed a high-fat diet. *Metab. Clin. Exp.* 2013;62(6): 851-60. doi:
441 10.1016/j.metabol.2012.12.007.

442 65. Basaria S, Muller DC, Carducci MA, Egan J, Dobs AS. Hyperglycemia and insulin
443 resistance in men with prostate carcinoma who receive androgen-deprivation therapy.
444 *Cancer.* 2006;106(3): 581-8. doi: 10.1002/cncr.21642.

445 66. Lubik AA, Gunter JH, Hendy SC, Locke JA, Adomat HH, Thompson V, et al. Insulin
446 increases de novo steroidogenesis in prostate cancer cells. *Cancer Res.* 2011;71(17):
447 5754-64. doi: 10.1158/0008-5472.can-10-2470.

448 67. Hsing AW, Sakoda LC, Chua S, Jr. Obesity, metabolic syndrome, and prostate cancer.
449 *Am. J. Clin. Nut.* 2007;86(3): s843-57.

450 68. Grossmann M, Wittert G. Androgens, diabetes and prostate cancer. *Endocrine-Related
451 Cancer.* 2012;19(5): F47-62. doi: 10.1530/erc-12-0067.

452 69. Maughan ML, Thomas PB, Crisp GJ, Philp LK, Shah ET, Herington AC, et al. Insights
453 from engraftable immunodeficient mouse models of hyperinsulinaemia. *Sci. Rep.*
454 2017;7(1): 491. doi: 10.1038/s41598-017-00443-x.

455 70. Scott KL, Kabbarah O, Liang M-C, Ivanova E, Anagnostou V, et al. GOLPH3
456 modulates mTOR signalling and rapamycin sensitivity in cancer. *Nature.* 2009; 459:
457 1085-1090.

458 71. Diabetes Trial Unit. The Oxford Centre for Diabetes EaM. HOMA Calculator University
459 of Oxford. Available from: <https://www.dtu.ox.ac.uk/homacalculator/index.php>.

460 72. Tong J, Davis HW, Summer S, Benoit SC, Haque A, Bidlingmaier M, et al. Acute
461 administration of unacylated ghrelin has no effect on Basal or stimulated insulin
462 secretion in healthy humans. *Diabetes.* 2014;63(7): 2309-19. doi: 10.2337/db13-1598.

463 73. Gortan Cappellari G, Zanetti M, Semolic A, Vinci P, Ruozzi G, Falcione A, et al.
464 Unacylated ghrelin reduces skeletal muscle reactive oxygen species generation and

465 inflammation and prevents high-fat diet-induced hyperglycemia and whole-body insulin
466 resistance in rodents. *diabetes*. 2016;65(4):874-86. doi: 10.2337/db15-1019.

467 74. Banks WA, Tschop M, Robinson SM, Heiman ML. Extent and direction of ghrelin
468 transport across the blood-brain barrier is determined by its unique primary structure. *J. Pharmacol. Exper. Therap.* 2002;302(2): 822-7. doi: 10.1124/jpet.102.034827.

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