Endocannabinoid receptor blockade increases vascular endothelial growth factor and inflammatory markers in obese women with PCOS

Running title: CB-1 blockade and VEGF in PCOS

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Abstract:

Context: Animal studies suggest that cannabinoid receptor-1 (CB-1) blockade reduces inflammation and neovascularization by decreasing vascular endothelial growth factor (VEGF) levels associated with a reduction in inflammatory markers, thereby potentially reducing cardiovascular risk.

Objective: To determine the impact of CB1 antagonism by rimonabant on VEGF and inflammatory markers in obese PCOS women.

Design: Randomised, open-labelled parallel study.

Setting: Endocrinology outpatient clinic in a referral centre.

Subjects: Twenty patients with PCOS and biochemical hyperandrogenaemia with a body mass index of $\geq 30 kg/m^2$ were recruited. Patients were randomised to 1.5g daily of metformin or 20mg daily of rimonabant.

Main Outcome Measures: Post hoc review to detect VEGF and pro-inflammatory cytokines TNF-α, IL-1β, IL-1ra, IL-2, IL6, IL-8, IL-10 and MCP-1 before and after 12 weeks treatment.

Results: After 12 weeks of rimonabant there was a significant increase in VEGF (99.2±17.6 vs 116.2±15.8pg/ml, $p<0.01$) and IL-8 (7.4±11.0 vs 18.1±13.2pg/ml, $p<0.05$) but not after metformin (VEGF $p=0.7$; IL-8 $p=0.9$). There was no significant difference in the pro-inflammatory cytokines TNF-α, IL-1β, IL-1ra, IL-2, IL6, IL-8, IL-10 and MCP-1 following either treatment.

Conclusion: This study suggests that rimonabant CB-I blockade paradoxically raised VEGF and the cytokine IL-8 in obese women with PCOS that may have offset the potential benefit associated with weight loss.

Introduction:

Vascular endothelial growth factor (VEGF) has a crucial role in inflammation and in vascular neogenesis, particularly in cancer [1, 2]. Animal studies have shown that cannabinoid receptor-1 (CB-1) blockade reduces inflammation and neovascularization by decreasing VEGF levels associated with a reduction in inflammatory markers [3]. Recent studies have shown that a reduction in VEGF levels may be associated with a better long term prognosis in cancer patients, with high VEGF levels being associated with a poorer prognosis [4, 5]. Weight loss induced by bariatric surgery has been shown to significantly reduce VEGF and other inflammatory cytokines, especially TNF-α, IL-1β, IL-1ra, IL-2, IL6, IL-8, IL-10 and MCP-1, potentially reducing cardiovascular risk [6-16]. In polycystic ovary syndrome (PCOS) patients, high serum and follicular fluid VEGF levels are associated with abnormal ovarian angiogenesis and dysfunction [17-20] and several studies have revealed improved ovarian function in these patients with decreasing VEGF levels [21-23]. Therefore, our hypothesis was that rimonabant, a CB-1 specific receptor antagonist, would reduce VEGF in PCOS by a decrease in weight and reduction in inflammatory cytokines and therefore such agents may be of potential benefit in the future. New human studies to look at this hypothesis could not be done as rimonabant was withdrawn due to side effects of depression and suicidal ideation and therefore a retrospective analysis of a previous study was undertaken [24].

Research design and methods:

We performed a post hoc analysis of a study whose primary endpoint was to investigate the effects of rimonabant on biochemical hyperandrogenaemia and insulin resistance in patients with PCOS. The secondary end-points were change in weight and waist circumference [24]. It was a randomized open labelled parallel study with rimonabant 20mg daily or metformin

(500mg three times daily) in 20 patients for 3 months: the study populations, designs, techniques and assays applied; along with informed written consent which have been described previously (Clinical trial registration ISRCTN75758249) [24]. All patients were women of Western European descent with a diagnosis of PCOS based on all three diagnostic criteria of the Rotterdam consensus, namely clinical and biochemical evidence of hyperandrogenaemia, oligomenorrhea or amenorrhoea and polycystic ovaries on transvaginal ultrasound [25] were recruited from Hull Royal Infirmary, UK. Study bloods and measurement were done after an overnight fast. Compliance was monitored by counting returned medication.

The Bio-Plex 200 system with HTF (Bio-Rad, Hercules, CA) was used to evaluate the sera and the levels of VEGF, TNF-α, IL-1β, IL-1ra, IL-2, IL6, IL-8, IL-10 and MCP-1 and compared to a set of standards that were run simultaneously in the assay.

Statistical analysis and sample size calculation:

The details of statistical methods used and power calculations have been explained previously in the original article [24]. The power of the study to demonstrate a significant reduction in total testosterone was based on a previous study showing a significant reduction in total testosterone concentration after treatment with metformin [26]. Using two-sided 5% significance level, a sample of 10 patients per group was found to be needed (assuming a 20% dropout rate) to detect changes in total testosterone with 90% power. Comparisons between the metformin group and the rimonabant-treated group, with respect to percentage changes from baseline were carried out using the paired t test for biochemical data and clinical observations. The Wilcoxon signed rank test was applied to biochemical data that violated the assumptions of normality when tested using the Kolmogorov-Smirnov test. For all analysis, a two-tailed $P \leq 0.05$ was considered to indicate statistical significance. Statistical

analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL), and nQuery version 4 was used for sample size determination.

Results:

All 20 subjects recruited completed the 3-month study period. The compliance was 98% in both groups by counting returned medication. The patients were weight matched in each group (Table 1).

Weight reduced significantly after 12 weeks of rimonabant treatment (104.6 ± 4.6 vs. 98.4 ± 4.7 kg, $p<0.01$) with a corresponding reduction in waist circumference (116.0 ± 3.3 vs. 109.2 ± 3.7 cm, $p<0.01$), hip circumference (128.5 ± 4.0 vs. 124.1 ± 4.2 cm, $p <0.03$) and waist hip ratio (0.90 ± 0.02 vs. 0.88 ± 0.01, $p <0.01$). In the metformin group these parameters were unchanged after treatment. There was a significant reduction from baseline in free androgen index (26.6 ± 6.1 vs. 16.6 ± 4.1 $p<0.01$) and testosterone (4.6 ± 0.4 vs. 3.1 ± 0.3 nmol/L $p<0.01$) but there was no significant reduction in the metformin treated group (free androgen index (FAI) $p=0.38$; testosterone $p=0.57$). The percentage reduction in testosterone was significantly higher in patients treated with rimonabant for 12 weeks compared to the metformin group (-33.2 ± 5.0 vs. -7.5 ± 1.0 % $p < 0.05$). There were no significant changes in sex hormone binding globulin (SHBG) in either group. HOMA-β and insulin reduced with rimonabant (288 ± 150 vs. 268 ± 217, $p< 0.05$ and 20 ± 7 vs. 16 ± 6, $p< 0.05$, respectively) whilst metformin showed no effect (217 ± 104 vs. 217 ± 120 and 15 ± 9 vs. 15 ± 11, respectively). Both treatments reduced glucose levels that reflected in a significant reduction of insulin resistance from baseline for rimonabant ($p<0.05$), but not with metformin, albeit that, the absolute change in HOMA-IR did not differ between the 2 groups. There was no significant improvement in any of the lipid parameters or for hsCRP within or between study groups [24].

There was a significant increase in VEGF (99.2±17.6 vs 116.2±15.8pg/ml, $p<0.01$) and IL-8 (7.4±11.0 vs 18.1±13.2pg/ml, $p<0.05$) after 12 weeks of rimonabant treatment but not after metformin (VEGF $p=0.7$; IL-8 $p=0.9$). There were no significant changes seen in the pro-inflammatory cytokines, TNF-α, IL-1β, IL-1ra, IL-2, IL6, IL-10 and MCP-1 following either treatment (Table 1).

Discussion:

This study showed that rimonabant increased VEGF and IL8 without affecting the other pro-inflammatory markers in obese women with PCOS that might not be beneficial in this group, counteracting the potential benefits associated with weight loss.

Angiogenesis is a vital aspect of normal ovarian cyclical function, essentially required for regular follicular development and growth. Studies have revealed that VEGF is the primary regulator of ovarian angiogenesis and VEGF pathway blockade is sufficient to disrupt ovarian angiogenesis and function [27]. PCOS is characterized by anomalous ovarian angiogenesis associated with increased ovarian expression and high follicular fluid concentration of VEGF, which is reflected in turn in their serum levels [28]. High levels of VEGF have been suggested to play an important role in PCOS pathophysiology and may contribute to ovarian dysfunction, subfertility and ovarian hyperstimulation syndrome (OHSS) [18, 29]. High VEGF levels have also been related directly to impaired insulin sensitivity in PCOS and reduced VEGF levels have been shown to cause significant improvement in glucose tolerance and insulin sensitivity in these patients and in diabetic and obese animal models, suggesting anti-VEGF therapy as a potentially novel treatment option in these patients [29-31]. Furthermore, significant reductions in VEGF levels have been seen in obese patients after weight loss, either by caloric restriction or by weight losing surgery, in addition to the significant improvements in metabolic parameters [32, 33]. However, in our

study CB-1 blockade with rimonabant increased VEGF levels in PCOS patients even though they showed significant weight loss and improved insulin sensitivity after 12 weeks of therapy. This increase in VEGF is likely to be a pharmacological effect of rimonabant given that one of the suggested mechanisms for high VEGF levels in PCOS patients is hyperinsulinaemia [34, 35] that was reduced by treatment in this study.

IL-8 levels were also significantly raised after rimonabant therapy that is a known proinflammatory and proangiogenic cytokine [36] and has been established as a risk factor for high cardiovascular risk [37, 38]. It has also been proposed to play a vital role in OHSS along with VEGF [39]. In vitro studies have shown that IL-8 enhances VEGF expression in endothelial cells by activating nuclear factor-kappa B (NF-kappa B) and stimulates hypoxia independent secretion of VEGF, which could aggravate ovarian dysfunction in PCOS patients by further stimulating abnormal ovarian angiogenesis [40]. Therefore, the increase in VEGF may be indirectly due to IL-8 stimulation.

Studies have shown that cannabinoids are NF-kappa B inhibitors, so one of the other possible mechanisms of increased VEGF in our study could be direct activation of NF-kappa B by CB-1 blockade after 12 weeks of rimonabant treatment [41].

In summary, our study suggests that CB-1 blockade caused by rimonabant paradoxically raises VEGF and the pro-inflammatory cytokine IL-8 in obese women with PCOS that may have a direct deleterious effect and offset the potential benefit associated with weight loss.

Table 1: **Comparison of cytokine profile before and after 12 weeks treatment with rimonabant or metformin**

Rimonabant Group (n = 10)				**Metformin Group** (n = 10)		
Cytokines	Baseline (V1)	12 Weeks (V2)	P value	Baseline (V1)	12 Weeks (V2)	P value
VEGF (pg/ml)	99.2±17.6	116.2±15.8	0.01*	110.3±25.2	111.5±24.8	0.7
IL-8 (pg/ml)	7.4±11.0	18.1±13.2	0.04*	12.3±13.2	11.2±13.8	0.9
TNF-α (pg/ml)	8.7±3.9	8.7±3.7	1.0	7.1±2.9	7.9±4.3	0.3
IL-1β (pg/ml)	85.1±76.7	91.5±91.1	0.3	86.2±55.7	105.2±88.8	0.2
IL-1RA (pg/ml)	576.9±126.5	609.7±118.5	0.4	592.5±211.3	683.7±418.2	0.3
IL-2 (pg/ml)	17.8±8.5	21.9±13.3	0.2	23.0±17.1	35.4±57.2	0.4
IL-6 (pg/ml)	16.8±8.4	18.5±9.5	0.3	12.2±5.9	13.2±7.3	0.3
IL-10 (pg/ml)	10.2±6.3	10.1±5.8	0.9	23.0±32.3	27.2±29.9	0.3
MCP-1 (pg/ml)	279.1±59.8	307.1±57.7	0.07	294.2±65.9	297.7±88.2	0.8

VEGF= Vascular Endothelial Growth Factor, TNF-α = Tumour Necrosis Factor Alpha, IL-1β = Interleukin-1Beta, IL-1RA = Interleukin-1 receptor antagonist, IL-2 = Interleukin-2, IL-6= Interleukin-6, IL-8 = Interleukin-8, IL-10= Interleukin-10, MCP-1= Monocyte Chemotactic Protein-1
Data are presented as mean ± SEM. ***:*** Significant difference from baseline.

References:

1. Waldner, M.J., et al., *VEGF receptor signaling links inflammation and tumorigenesis in colitis-associated cancer.* J Exp Med, 2010. **207**(13): p. 2855-68.
2. Goel, H.L. and A.M. Mercurio, *VEGF targets the tumour cell.* Nat Rev Cancer, 2013. **13**(12): p. 871-82.
3. Guabiraba, R., et al., *Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation and neovascularization in a model of sponge-induced inflammatory angiogenesis.* Inflamm Res, 2013. **62**(8): p. 811-21.
4. Tara Beth Sanft, B.C., Maura Harrigan, Fangyong Li, Erikka Loftfield, Mary Playdon, Yang Zhou, Cary Philip Gross, Jennifer A. Ligibel, KATHRYN H. SCHMITZ, Dawn L. Hershman, Lajos Pusztai, Melinda L Irwin, *Impact of weight loss and exercise on VEGF levels in breast cancer survivors.* J Clin Oncol 34, 2016 (suppl; abstr 10103), 2016.
5. Krzystek-Korpacka, M., et al., *Impact of weight loss on circulating IL-1, IL-6, IL-8, TNF-alpha, VEGF-A, VEGF-C and midkine in gastroesophageal cancer patients.* Clin Biochem, 2007. **40**(18): p. 1353-60.
6. Haggerty, A.F., et al., *The use of novel technology-based weight loss interventions for obese women with endometrial hyperplasia and cancer.* Gynecol Oncol, 2016. **140**(2): p. 239-44.
7. Shih, K.C., et al., *Effects of bariatric weight loss surgery on glucose metabolism, inflammatory cytokines, and serum tartrate-resistant acid phosphatase 5a in obese Chinese adults.* Clin Chim Acta, 2016. **453**: p. 197-202.
8. Sams, V.G., et al., *Effect of bariatric surgery on systemic and adipose tissue inflammation.* Surg Endosc, 2015.
9. Lindegaard, K.K., et al., *Effects of Roux-en-Y gastric bypass on fasting and postprandial inflammation-related parameters in obese subjects with normal glucose tolerance and in obese subjects with type 2 diabetes.* Diabetol Metab Syndr, 2015. **7**: p. 12.
10. Mallipedhi, A., et al., *Changes in inflammatory markers after sleeve gastrectomy in patients with impaired glucose homeostasis and type 2 diabetes.* Surg Obes Relat Dis, 2014. **10**(6): p. 1123-8.
11. Montecucco, F., et al., *Gastric bypass in morbid obese patients is associated with reduction in adipose tissue inflammation via N-oleoylethanolamide (OEA)-mediated pathways.* Thromb Haemost, 2015. **113**(4): p. 838-50.
12. Netto, B.D., et al., *Roux-en-Y gastric bypass decreases pro-inflammatory and thrombotic biomarkers in individuals with extreme obesity.* Obes Surg, 2015. **25**(6): p. 1010-8.
13. He, B., et al., *Roux-en-Y esophagojejunostomy reduces serum and aortic inflammatory biomarkers in type 2 diabetic rats.* Obes Surg, 2014. **24**(6): p. 916-26.
14. Thomsen, S.B., et al., *Effects of Roux-en-Y gastric bypass on fasting and postprandial levels of the inflammatory markers YKL-40 and MCP-1 in patients with type 2 diabetes and glucose tolerant subjects.* J Obes, 2013. **2013**: p. 361781.
15. Rao, S.R., *Inflammatory markers and bariatric surgery: a meta-analysis.* Inflamm Res, 2012. **61**(8): p. 789-807.
16. Dalmas, E., et al., *Variations in circulating inflammatory factors are related to changes in calorie and carbohydrate intakes early in the course of surgery-induced weight reduction.* Am J Clin Nutr, 2011. **94**(2): p. 450-8.
17. Abramovich, D., et al., *Angiopoietins/TIE2 system and VEGF are involved in ovarian function in a DHEA rat model of polycystic ovary syndrome.* Endocrinology, 2012. **153**(7): p. 3446-56.
18. Tal, R., D.B. Seifer, and A. Arici, *The emerging role of angiogenic factor dysregulation in the pathogenesis of polycystic ovarian syndrome.* Semin Reprod Med, 2015. **33**(3): p. 195-207.

19. Savchev, S.I., et al., *Follicular fluid-specific distribution of vascular endothelial growth factor isoforms and sFlt-1 in patients undergoing IVF and their correlation with treatment outcomes.* Reprod Sci, 2010. **17**(11): p. 1036-42.
20. Peitsidis, P. and R. Agrawal, *Role of vascular endothelial growth factor in women with PCO and PCOS: a systematic review.* Reprod Biomed Online, 2010. **20**(4): p. 444-52.
21. Di Pietro, M., et al., *Metformin regulates ovarian angiogenesis and follicular development in a female polycystic ovary syndrome rat model.* Endocrinology, 2015. **156**(4): p. 1453-63.
22. El Behery, M.M., et al., *Effect of laparoscopic ovarian drilling on vascular endothelial growth factor and ovarian stromal blood flow using 3-dimensional power Doppler.* Int J Gynaecol Obstet, 2011. **112**(2): p. 119-21.
23. Amin, A.F., et al., *Evaluation of the impact of laparoscopic ovarian drilling on Doppler indices of ovarian stromal blood flow, serum vascular endothelial growth factor, and insulin-like growth factor-1 in women with polycystic ovary syndrome.* Fertil Steril, 2003. **79**(4): p. 938-41.
24. Sathyapalan, T., et al., *A comparison between rimonabant and metformin in reducing biochemical hyperandrogenaemia and insulin resistance in patients with polycystic ovary syndrome (PCOS): a randomized open-label parallel study.* Clinical Endocrinology, 2008. **69**(6): p. 931-935.
25. *Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS).* Hum Reprod, 2004. **19**(1): p. 41-7.
26. Jayagopal, V., et al., *Orlistat is as beneficial as metformin in the treatment of polycystic ovarian syndrome.* J Clin Endocrinol Metab, 2005. **90**(2): p. 729-33.
27. Ferrara, N., *Vascular endothelial growth factor: basic science and clinical progress.* Endocr Rev, 2004. **25**(4): p. 581-611.
28. Scotti, L., et al., *Platelet-derived growth factor BB and DD and angiopoietin1 are altered in follicular fluid from polycystic ovary syndrome patients.* Mol Reprod Dev, 2014. **81**(8): p. 748-56.
29. Cheng, F., et al., *Serum vascular endothelial growth factor B is elevated in women with polycystic ovary syndrome and can be decreased with metformin treatment.* Clin Endocrinol (Oxf), 2016. **84**(3): p. 386-93.
30. Hagberg, C.E., et al., *Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes.* Nature, 2012. **490**(7420): p. 426-30.
31. Honek, J., et al., *Modulation of age-related insulin sensitivity by VEGF-dependent vascular plasticity in adipose tissues.* Proc Natl Acad Sci U S A, 2014. **111**(41): p. 14906-11.
32. Cullberg, K.B., et al., *Effect of weight loss and exercise on angiogenic factors in the circulation and in adipose tissue in obese subjects.* Obesity (Silver Spring), 2013. **21**(3): p. 454-60.
33. Garcia de la Torre, N., et al., *Effects of weight loss after bariatric surgery for morbid obesity on vascular endothelial growth factor-A, adipocytokines, and insulin.* J Clin Endocrinol Metab, 2008. **93**(11): p. 4276-81.
34. Mick, G.J., X. Wang, and K. McCormick, *White adipocyte vascular endothelial growth factor: regulation by insulin.* Endocrinology, 2002. **143**(3): p. 948-53.
35. Hale, L.J., et al., *Insulin directly stimulates VEGF-A production in the glomerular podocyte.* Am J Physiol Renal Physiol, 2013. **305**(2): p. F182-8.
36. Koch, A.E., et al., *Interleukin-8 as a macrophage-derived mediator of angiogenesis.* Science, 1992. **258**(5089): p. 1798-801.
37. Apostolakis, S., et al., *Interleukin 8 and cardiovascular disease.* Cardiovasc Res, 2009. **84**(3): p. 353-60.
38. Romuk, E., et al., *Selectin-P and interleukin-8 plasma levels in coronary heart disease patients.* Eur J Clin Invest, 2002. **32**(9): p. 657-61.

39. Chen, S.U., et al., *Signal mechanisms of vascular endothelial growth factor and interleukin-8 in ovarian hyperstimulation syndrome: dopamine targets their common pathways.* Hum Reprod, 2010. **25**(3): p. 757-67.
40. Martin, D., R. Galisteo, and J.S. Gutkind, *CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex.* J Biol Chem, 2009. **284**(10): p. 6038-42.
41. Juttler, E., et al., *The cannabinoid dexanabinol is an inhibitor of the nuclear factor-kappa B (NF-kappa B).* Neuropharmacology, 2004. **47**(4): p. 580-92.