Influence of rimonabant treatment on peripheral blood mononuclear cells; flow cytometry analysis and gene expression profiling

Stefan Almestrand, Xiao Wang, Åsa Jeppsson-Ahlberg, Marcus Nordgren, Jenny Flygare, Birger Christensson, Stephan Rössner, Birgitta Sander

The cannabinoid receptor type 1 (CB1) antagonist rimonabant has been used as treatment for obesity. In addition, anti-proliferative effects on mitogen-activated leukocytes have been demonstrated in vitro. We have previously shown that rimonabant (SR141716A) induces cell death in ex vivo isolated malignant lymphomas with high expression of CB1 receptors. Since CB1 targeting may be part of a future lymphoma therapy it was of interest to investigate possible effects on peripheral blood mononuclear cells (PBMC) in patients treated with rimonabant. We therefore evaluated leukocyte subsets by 6 color flow cytometry in eight patients before and at treatment with rimonabant for 4 weeks. Wholetranscript gene expression profiling in PBMC before and at 4 weeks of rimonabant treatment was done using Affymetrix Human Gene 1.0 ST Arrays. Our data show no significant changes of monocytes, B cells, total T cells or T cell subsets in PBMC during treatment with rimonabant. There was a small but significant increase in NK cells after rimonabant therapy. Gene expression analysis detected significant changes in expression of genes associated with innate immunity, cell death and metabolism. The present study shows that normal monocytes and leukocyte subsets in blood remain rather constant during rimonabant treatment. This is in contrast to the induction of cell death previously observed in CB1 expressing lymphoma cells in response to treatment with rimonabant in vitro. These differential effects observed on normal and malignant lymphoid cells warrant investigation of CB1 targeting as a potential lymphoma treatment.

- Influence of rimonabant treatment on peripheral blood mononuclear cells; flow cytometry analysis and
 gene expression profiling.
- 4 Abstract

The cannabinoid receptor type 1 (CB1) antagonist rimonabant has been used as treatment for obesity. 5 In addition, anti-proliferative effects on mitogen-activated leukocytes have been demonstrated in vitro. 6 We have previously shown that rimonabant (SR141716A) induces cell death in ex vivo isolated 7 8 malignant lymphomas with high expression of CB1 receptors. Since CB1 targeting may be part of a future lymphoma therapy it was of interest to investigate possible effects on peripheral blood 9 mononuclear cells (PBMC) in patients treated with rimonabant. We therefore evaluated leukocyte 10 11 subsets by 6-color flow cytometry in eight patients before and at treatment with rimonabant for 4 weeks. Gene expression profiling in PBMC before and at 4 weeks of rimonabant treatment was done 12 13 using Affymetrix Human Gene 1.0 ST arrays. Our data show no significant changes of B cells, total T 14 cells or T cell subsets in PBMC during treatment with rimonabant. There was a small but significant increase in NK cells. Gene expression analysis detected significant changes in expression of genes 15 16 associated with innate immunity, cell death and metabolism. The present study shows that leukocyte 17 subsets in blood remain rather constant during rimonabant treatment. This is in contrast to the induction of cell death previously observed in CB1 expressing lymphoma cells in response to treatment 18 19 with rimonabant in vitro. These differential effects observed on normal and malignant lymphoid cells 20 warrant investigation of CB1 targeting as a potential lymphoma treatment.

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Stefan Almestrand^{1, 2)}, XiaoWang^{1, 3)}, Åsa Jeppson-Ahlberg⁴⁾, Marcus Nordgren^{1, 5)}, Jenny
 Flygare^{1, 6)}, Birger Christensson¹⁾, Stephan Rössner⁷⁾ and Birgitta Sander^{1*)}

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| 26 | 1) Department | of Laboratory | Medicine, | Division of | of Pathology, | Karolinska | Institutet | and Karc | linska |
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|----|---------------|---------------|-----------|-------------|---------------|------------|------------|----------|--------|

- 27 University Hospital Huddinge, Stockholm, Sweden
- 28 2) Present address: AstraZeneca, Södertälje, Sweden
- 29 3) Present address: Center for Primary Health Care Research, Skåne University Hospital, Malmö,

30 Sweden

- 31 4) Pathology/Cytology, Karolinska University Hospital Huddinge, Stockholm, Sweden
- 32 5) Present address: Laboratory of Lipid Biochemistry and Protein Interactions, Department of Cellular
- 33 and Molecular Medicine, KU Leuven, Leuven, Belgium
- 34 6) Present address: Department of Laboratory Medicine, Division of Clinical Chemistry, Karolinska
- 35 Institutet, Stockholm Sweden
- 36 7) Department of Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden
- 37 *) Corresponding author
- 38
- 39
- 40
- 41 Corresponding author: Birgitta Sander, Dept. of Laboratory Medicine, Div. of Pathology, F46,
- 42 Karolinska Institutet and Karolinska University Hospital Huddinge, SE 141 86 Stockholm, Sweden.
- 43 Email: <u>birgitta.sander@ki.se</u>
- 44 Tel: +46 8 58581044 Fax: +46 8 58581020
- 45 Introduction
- 46
- 47 The endocannabinoid system consists of the cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2)
- 48 receptors, their endogenous ligands anandamide and 2-arachidonoyl glycerol and the enzymes involved
- 49 in their biosynthesis and metabolism (Di Marzo et al. 2004). CB1 is involved in the regulation of food

50 intake, energy balance and metabolism of glucose and lipids (Di Marzo & Matias 2005). In clinical 51 studies CB1 receptor blockage by the selective CB1 antagonist rimonabant (SR141716A) induced 52 weight loss and improvement in serum lipid, glucose and insulin levels by targeting central and 53 peripheral CB1 receptors (Van Gaal et al. 2008). However, some patients experienced depressions and 54 this was considered an unacceptable side effect for treating obesity/metabolic syndrome. Hence the drug was withdrawn from clinical use but there is remaining interest in some of its many potential 55 medical applications (Cooper & Regnell 2014; Zhou et al. 2012) including treatment of various 56 57 malignancies. It is therefore of interest to investigate possible adverse effects on blood cells in patients treated with rimonabant. 58 59 The endocannabinoid system is regulating various aspects of lymphocyte proliferation, maturation and immune response (Klein 2005; Muppidi et al. 2011; Pandey et al. 2009; Pereira et al. 2009; Sido et al. 60 2014). Targeting the endocannabinoid system may therefore be a possible new treatment option in 61 62 various lymphoproliferative disorders. CB1 receptors are expressed on cells of the immune system, but 63 generally at lower levels than CB2 (Bouaboula et al. 1993; Galiegue et al. 1995). We and others have found that CB1 and CB2 are highly expressed on neoplastic lymphocytes in malignant lymphoma 64 65 (Gustafsson et al. 2008; Islam et al. 2003; McKallip et al. 2002; Wasik et al. 2014). Targeting of CB1 and CB2 with endogenous or synthetic agonists reduced cell proliferation in vitro and in vivo and 66 induced programmed cell death selectively in tumor cells of mantle cell lymphoma (Flygare et al. 67 2005; Gustafsson et al. 2006; Gustafsson et al. 2008; Wasik et al. 2011a). Similarly, CB2 agonists 68 induced cell death in T cell lymphoblastic leukemia (McKallip et al. 2002). Also the CB1 antagonist 69 rimonabant impaired proliferation and induced cell death in ex vivo isolated mantle cell lymphoma 70 cells, alone, or in combination with anandamide (Flygare et al. 2005). Others have reported 71 72 antiproliferative effects of rimonabant on in vitro activated PBMC but not on freshly isolated, non-73 activated PBMC (Gallotta et al. 2010; Malfitano et al. 2008). These results show that CB1 blockade

| 74 | may have immunomodulatory and antiproliferative effects on malignant lymphoma and on activated |
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| 75 | normal lymphocytes in vitro but seems to spare resting lymphocytes. Very little is published on effects |
| 76 | of rimonabant on human PBMC in vivo and the aim of this study was to investigate how treatment with |
| 77 | rimonabant affected blood leukocytes. We therefore collected blood cells from obese patients treated |
| 78 | with rimonabant and analyzed blood leukocytes by flow cytometry before and during treatment. To |
| 79 | investigate which genes were differentially expressed in PBMC during rimonabant treatment we used |
| 80 | oligonucleotide arrays to compare gene expression profiles in PBMC before and at 4 weeks of |
| 81 | treatment. This pilot study shows that rimonabant treatment induces expression of genes involved in |
| 82 | immune responses but have only marginal effects on leukocyte subset frequencies in blood. |
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| 89 | Materials and Methods |
| 90 | Patients and study design: |
| 91 | Rimonabant was prescribed to eight patients, admitted to the Overweight Study Unit at the Department |
| 92 | of Medicine, Karolinska University Hospital. Rimonabant was administered according to the |
| 93 | manufacturers guidelines. All patients had a $BMI > 35 \text{ kg/m}^2$, were treated on clinical indications |
| 94 | (metabolic and mechanical disability) and without mental disturbances. They were not included in any |
| 95 | other study. The clinical characteristics of these patients are presented in Table 1. Blood samples were |
| 96 | collected before treatment and at the first clinical control, when the patients had received rimonabant, |
| 97 | 20 mg daily, for 4 weeks. All patients gave their informed consent and the study was performed in |

98 accordance with the Declaration of Helsinki and approved by the Regional Ethical Committee in99 Stockholm.

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101 Flow cytometry:

102 The phenotypes of cells in the blood were analyzed by flow cytometry according to standard procedures at the Hematopathology Unit, Dept. of Pathology, Karolinska University Hospital, using 6 103 104 color flow cytometry to detect T, B, NK cells and CD3- CD4+ cells (monocytes and dendritic cells). 105 Flow cytometry was performed on a CANTO 1 flow cytometer (BD, Becton-Dickinson, Europe). 106 For data acquisition and analysis, a CANTO 1 flow cytometer (BD, Becton Dickinson, Europe) was 107 used with Cell Quest software (Becton Dickinson). All samples were analyzed by setting appropriate 108 side and forward scatter gates to identify the mononuclear cell population, using CD45 and forward 109 and side scatter for gate setting. Consistency of analysis parameters was ascertained by calibrating the 110 flow cytometer with calibrating beads and FacsComp software, both from Becton Dickinson. The 111 results are reported as percentage of gated cells positive for each antibody. The following fluorochrome 112 conjugated antibodies, all from BD, were used: CD4 PE, CD3 PerCP-Cy5.5, CD19 PE-Cy7, CD8 APC 113 and CD45 APC-H7. We also used BD Multitest 6-Color TBNK Reagent containing CD3 FITC clone SK7, CD16 PE clone B73, CD56 PE clone NCAM 16.2, CD45 PerCP-Cy5.5 clone 2D1, CD4 PE-Cy7 114 115 clone SK3, CD19 APC clone SJ25C1 and CD8 APC-Cy7, clone SK1.

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117 <u>RNA isolation and oligonucleotide array hybridization:</u>

118 Blood mononuclear cells were isolated by Ficoll separation (Ficoll-Paque PLUS, GE Healthcare).

119 From the cell-pellet total RNA was prepared using Qiagen midi plus kit (Qiagen GmbH, Hilden

120 Germany) as recommended by the manufacturer and was quality controlled on an Agilent Bioanalyzer

121 (Agilent Technologies, Inc. Palo Alto, CA). Six pretreatment samples and seven samples obtained after

| 122 | rimonabant treatment passed the quality control. The cRNA synthesis for microarray experiments and |
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| 123 | the hybridizations were carried out using Affymetrix Human Gene 1.0 ST Array (Affymetrix, Inc., |
| 124 | Santa Clara, CA) according to standard Affymetrix protocols at the core facility for Bioinformatics and |
| 125 | Expression Analysis, Department of Biosciences and Nutrition, Karolinska Institutet. |
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| 127 | Gene expression data analysis: |
| 128 | We used tools provided in the Partek Genomic Suite 6.5 software (Partek Inc., St. Louis, MO). |
| 129 | Normalization was done by Robust Multiarray Analysis (RMA) followed by 1-way Analysis Of |
| 130 | Variance (ANOVA) comparing the patient group before and after treatment. Significantly changed |
| 131 | genes and exons were selected with an unadjusted p-value of <0,001, a False Discovery Rate (FDR) |
| 132 | <0,1 and a fold-change equal or greater than $>1,5$ for up regulated genes and equal or less than $<-1,5$ |
| 133 | for down regulated genes. Gene functional annotations were performed by using the free software |
| 134 | DAVID v6.7 (Database for Annotation, Visualization and Integrated Discovery) (Huang da et al. |
| 135 | 2009). The gene expression data are deposited at the GEO repository under the number GSE68055. |
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| 138 | Statistical analysis |
| 139 | Leukocyte subsets (as measured by flow cytometry) in blood before and after rimonabant treatment |
| 140 | were analyzed using a paired t-test. |
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| 143 | Results |
| 144 | Analysis of PBMC by flow cytometry before and during treatment with rimonabant |

Blood levels of mononuclear cells on eight obese patients were analyzed by flow cytometry before and during treatment with rimonabant. There were no significant changes in the relative frequencies of total CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells or CD3-CD4+ cells (monocytes and dendritic cells) in the patients during the treatment period (Table 1, graphically presented in Fig.1) There was however a trend towards an increase in percentage of NK cells (before treatment median 9% range 7-14%; after treatment median 12% range 9-15% p=0,049) (Table 1, Fig. 1).

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Whole-transcript gene expression analysis demonstrates significant changes in genes belonging to
innate immune system pathways.

154 Treatment with rimonabant might influence the expression of genes in patient leukocytes. To explore possible differences in gene expression profiles, whole-transcript expression analysis of PBMC 155 156 before and during treatment was done, using Affymetrix Human Gene 1.0 ST Arrays. 47 probe sets 157 were significantly differently expressed during treatment with a fold change of at least 1,5, 37 probe 158 sets showed increased expression and 10 decreased expression, respectively (Table 1). Several of the 159 genes with significantly increased expression after rimonabant treatment are known components of the 160 innate immune system (as exemplified by KLRF1, LILRA2, CTSB, CD160, CD177, and LY96). 161 KLRF1 (also named NKp80) encodes a lectin-type of receptor that is expressed on nearly all NK cells 162 and stimulates their cytotoxicity and cytokine release (Kuttruff et al. 2009). LILRA2 is the gene for an immune receptor that is expressed on monocytes, B cells, NK cells and dendritic cells and affects 163 antigen presentation and innate immune responses (Lu et al. 2012). CTSB encodes cathepsin B, a 164 165 protein that can be expressed in several immune cells including monocytes and that is involved in cell migration and immune modulation (Staun-Ram & Miller 2011). CD160 is an essential NK cell receptor 166 and is involved in regulation of cytokine production (reviewed in (Le Bouteiller et al. 2011). CD177 is 167 168 a GPI linked cell surface molecule that regulates activation and migration of neutrophil granulocytes

169 (Stroncek 2007). LY96 (also named MD-2) is associating with toll-like receptor 4 and is involved in 170 signaling by LPS (Mancek-Keber & Jerala 2015). A few genes promoting increased apoptosis were also upregulated (BCL-like 13, an apoptosis facilitator (Jensen et al. 2014; Kataoka et al. 2001), 171 172 RING1- and YY1-binding protein, a regulator of MDM2 (Chen et al. 2009)). It has previously been shown that chronic marijuana users have increased expression of CB1 in 173 peripheral blood mononuclear cells (Nong et al. 2002). We therefore specifically analyzed the 174 expression of genes belonging to the endocannabinoid system in our patient cohort. Rimonabant 175 176 treatment did neither affect the expression of CB1 (mean and standard deviation of CB1 expression values before and after treatment were 12,5 +/- 3,24 and 10,79 +/- 2,43, respectively, corresponding to 177 178 a fold change of -1,1) nor of CB2 or the enzymes involved in the degradation and/or synthesis of endocannabinoids (fatty acid amide hydrolase, FAAH, and N-acyl phosphatidylethanolamine 179 phospholipase D, NAPEPLD) either when analyzed by gene expression analysis or by RT-PCR (data 180

181 not shown).

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183 **Discussion**

184 In this study we investigated the possible effects on PBMC of treatment with the CB1 antagonist rimonabant in patients taking the drug for obesity. We found that the distribution of leukocyte subsets 185 186 remained rather constant, as analyzed by flow cytometry before treatment and after 4 weeks of treatment with rimonabant with the exception of NK cells that increased after treatment. There were no 187 188 significant changes in expression levels of cannabinoid receptors or enzymes involved in synthesis and 189 metabolism of endocannabinoids. However gene expression analysis suggested that genes involved in metabolism, cell death and the innate immune system were up regulated during treatment. 190 191 Rimonabant is the first selective CB1 antagonist registered for clinical use and was clinically

193 proliferative actions on normal and malignant cells have been reported. Cannabinoid receptors are 194 often more highly expressed on malignant cells than on their normal counterparts and cancer cells are 195 usually more sensitive to the action of cannabinoids than normal cells (reviewed in (Flygare & Sander 196 2007; Sido et al. 2014; Wasik et al. 2011b)). Rimonabant has been reported to induce growth inhibition 197 or apoptosis on several malignancies including breast, thyroid and colon cancer (Bifulco et al. 2004; De Petrocellis et al. 1998; Santoro et al. 2009; Sarnataro et al. 2006). We have previously 198 demonstrated that mantle cell lymphoma and other B cell lymphomas have higher expression of CB1 199 and CB2 than normal lymphocytes (Gustafsson et al. 2008; Islam et al. 2003; Wasik et al. 2014). 200 Cannabinoid receptor agonists, at 1-10 µM levels, reduced proliferation and induced programmed cell 201 202 death in mantle cell lymphoma in vitro and in a xenotransplant model (Flygare et al. 2005; Gustafsson et al. 2006; Gustafsson et al. 2008; Schatz et al. 1997; Wasik et al. 2011a). Interestingly, similar 203 concentrations of rimonabant induced cell death in *ex vivo* isolated mantle cell lymphoma cells 204 205 (Flygare et al. 2005). While these studies suggest that targeting of CB1 may be of use in cancer therapy, concern may be raised since anti-proliferative effects have been reported in PBMC (Malfitano 206 et al. 2008). In these studies rimonabant inhibited mitogen induced cell proliferation in vitro via G1/S 207 phase arrest without induction of cell death (Malfitano et al. 2008). In contrast, Gallotta et al. reported 208 209 that freshly isolated PBMC are highly resistant to the cytotoxic and cytostatic effects of rimonabant 210 compared to leukemia-derived cell lines (Gallotta et al. 2010). Possibly, the different sensitivity to CB1 antagonism in freshly isolated, compared to mitogen activated, PBMC may reflect differences in 211 212 expression levels of CB1. Resting leukocytes express very low levels of CB1 (Bouaboula et al. 1993; Galiegue et al. 1995; Kaminski et al. 1992) while receptor levels may increase upon activation by 213 mitogens, cytokines or exposure to CB1 agonists (Borner et al. 2007; Nong et al. 2002; Schatz et al. 214 1997). We did not detect any significant differences in expression levels of CB1 or other components 215

216 of the endocannabinoid system during rimonabant treatment for 4 weeks. Furthermore, our studies on 217 ex vivo isolated PBMC from rimonabant treated patients could not demonstrated very minor changes in frequencies of T cells, B cells, NK cells or CD3-CD4+ cells or on total lymphocyte counts, in line with 218 219 the results of Gallotta et al. (Gallotta et al. 2010). Global gene expression analysis demonstrated significant changes in genes coding for components of 220 the innate immune system. The study design does not make it possible to discriminate if the 221 222 differences in gene expression can be ascribed to certain subsets of leukocytes or if it is a general 223 process, seen in all PBMC. However, many of the genes that were more highly expressed after 224 treatment with rimonabant are expressed in NK cells (such as KLRF1 and CD160) and monocytes, 225 which imply that the treatment is associated with the activation of certain inflammatory and immunological functions of the innate immune system. Interestingly, rimonabant has been shown to 226 227 directly activate human and mouse macrophages and thereby inhibit the development of the 228 intracellular pathogen Brucella suis (Gross et al. 2000). Furthermore, studies on lipopolysaccharide activated human macrophages showed that CB1 receptor blockade by rimonabant suppressed 229 230 production of inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α) and matrix metalloproteinase-9 (Sugamura et al. 2009). 231

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234 Conclusions

In conclusion our results show that rimonabant treatment induces expression of genes involved in
immune responses but have only marginal effects on leukocyte subset frequencies in blood. This is in
marked contrast to previous studies in which rimonabant induced cell death in malignant B
lymphocytes that express high levels of CB1 (Flygare et al. 2005). The relatively small effects on

- 239 normal leukocytes suggest that CB1 targeting may be further investigated as a therapeutic approach in
- 240 lymphoma treatment, enabling selective effects of tumor cells.
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361 Legends to tables

- Table 1. Clinical parameters of included subjects and percentages of blood cells as analyzed by flow
- 363 cytometry before and after 4 weeks of rimonabant treatment.
- 364 # Values are percentage of cells in mononuclear gate. Total T cells were defined as CD3+, CD4+ T
- cells as CD3+CD4+, CD8+ T cells as CD3+CD8+ and B cells as CD19+. The CD3-CD4+ cell
- 366 population consists of monocytes and dendritic cells. NK cells were defined as CD3-, CD16+ and/or

367 CD56+. There was a significant increase in NK cells after treatment (p=0,049, paired t-test), all other
 368 changes were non significant.

369

Table 2. Genes differentially expressed in PBMC after treatment with rimonabant (ratio >1,5 between
rimonabant treated and controls, p-value <0,001, false discovery rate <0,1).

372

373 Figure legends

374

375 Figure 1. Percentage of peripheral blood mononuclear cells (PBMC) before and during treatment with 376 rimonabant. PBMC were analyzed by flow cytometry before start of therapy and 4 weeks later and 377 results are given as percentage of mononuclear cells in blood. Each data point represents results from 378 one patient. In cases with no change in the frequency of a certain cell type the data point would fall on the line. The only statistically significant change was for NK cells (p=0,049). For the other subsets the 379 p-values were as follows: CD3+ p=0.47: CD3+CD4+ p=0.25: CD3+CD8+ p=0.11: CD19+ p=0.13: 380 CD3-CD4+ p=0,11. The mononuclear gate was defined by CD45 in combination with side and forward 381 scatter. Within this gate the frequencies of T cells (CD3+), B cells (CD19+), NK cells (CD3-, CD56+ 382 383 and/or CD16+) and monocytes and dendritic cells (CD3-, CD4+) were analyzed. 384 385 386 387 388

Table 1(on next page)

Clinical parameters of included subjects and percentages of blood cells as analyzed by flow cytometry before and after 4 weeks of rimonabant treatment.

Values are percentage of cells in mononuclear gate. Total T cells were defined as CD3+, CD4+ T cells as CD3+CD4+, CD8+ T cells as CD3+CD8+ and B cells as CD19+. The CD3-CD4+ cell population consists of monocytes and dendritic cells. NK cells were defined as CD3-, CD16+ and/or CD56+. There was a significant increase in NK cells after treatment (p=0,049, paired t-test), all other changes were non significant.

| | Age, sex | Weight change (kg) | Leukocyte subsets as analyzed by flow cytometry# | | | | | | | | | | | |
|---------|---------------|--------------------------|--|---------|--------------|---------|--------------|---------|---------|--------|----------------|------------------|----------|--------|
| Dationt | | | Total T cells | | CD4+ T cells | | CD8+ T cells | | B cells | | CD3-CD4+ cells | | NK cells | |
| Fatient | | | before | after | before | after | before | after | before | after | before | after | before | after |
| 1 | 50, F | nd | 75 | 73 | 61 | 59 | 14 | 13 | 15 | 12 | 7,2 | 8 | 9 | 14 |
| 2 | 41, F | -6,3 | 58 | 61 | 37 | 34 | 21 | 26 | 32 | 25 | 7 | 6,6 | 8 | 12 |
| 3 | 55, F | nd | 72 | 72 | 54 | 53 | 19 | 20 | 16 | 12 | 5,7 | 8 | 11 | 15 |
| 4 | 56 <i>,</i> M | -3,1 | 70 | 72 | 48 | 51 | 21 | 20 | 15 | 17 | 5 <i>,</i> 9 | 4,6 | 14 | 10 |
| 5 | 47, F | -2,8 | 75 | 73 | 51 | 49 | 23 | 24 | 11 | 12 | 4,9 | 8 | 13 | 15 |
| 6 | 44, F | 0,0 | 69 | 70 | 51 | 50 | 17 | 19 | 20 | 18 | 3,9 | 4,6 | 8 | 12 |
| 7 | 69, F | -2,0 | 81 | 81 | 37 | 35 | 45 | 46 | 8 | 8 | 3,3 | 3,4 | 9 | 11 |
| 8 | 58, M | -3,0 | 85 | 87 | 52 | 53 | 32 | 40 | 4 | 3 | 2,1 | 5,7 | 7 | 9 |
| | | | | | | | | | | | | | | |
| median | 52,5 | 2,9 | 73,5 | 72,5 | 51 | 50,5 | 21 | 22 | 15 | 12 | 5,3 | 6,15 | 9 | 12 |
| | | | | | | | | | | | | | | |
| (range) | (41-69) | (0-6,3) | (58-85) | (61-87) | (37-61) | (34-59) | (14-45) | (13-46) | (4-32) | (3-25) | (2,1-7,2) | (3 <i>,</i> 4-8) | (8-14) | (9-15) |

Table 2(on next page)

Genes differentially expressed in PBMC after treatment with rimonabant (ratio >1,5 between rimonabant treated and controls, p-value <0,001, false discovery rate <0,1).

| 2 | |
|---|--|
| 3 | |

| Probe set | Gene symbol | Fold | p-value | Gene name |
|-----------|-------------|--------|---------|---|
| ID | | change | | |
| | | | | killer cell lectin-like receptor subfamily F, |
| 7953892 | KLRF1 | 2.50 | 0.00046 | member 1 |
| 7983910 | AQP9 | 2.25 | 0.00033 | aquaporin 9 |
| | | | | leukocyte immunoglobulin-like receptor, |
| 8031207 | LILRA2 | 2.10 | 0.00059 | subfamily A |
| 8078008 | LSM3 | 2.00 | 0.00068 | LSM3 homolog |
| 7981290 | WARS | 1.99 | 0.00028 | tryptophanyl-tRNA synthetase |
| 8149330 | CTSB | 1.90 | 0.00089 | cathepsin B |
| 8127534 | C6orf150 | 1.87 | 0.00035 | |
| 7919243 | CD160 | 1.79 | 0.00096 | CD160 molecule |
| 8130732 | BRP44L | 1.74 | 0.00073 | brain protein 44-like |
| 8110318 | PRELID1 | 1.72 | 0.00084 | PRELI domain containing 1 |
| 8003953 | PSMB6 | 1.69 | 0.00013 | proteasome subunit, beta type, 6 |
| 8015545 | RAB5C | 1.68 | 0.00085 | RAB5C, member RAS oncogene family |
| 8133690 | MDH2 | 1.68 | 0.00081 | malate dehydrogenase 2 |
| 8178676 | NEU1 | 1.67 | 0.00052 | sialidase 1 |
| 7973110 | RNASE2 | 1.65 | 0.00028 | ribonuclease, RNase A family, 2 |
| 026541 | FAM32A | 1.65 | 0.00032 | family with sequence similarity 32, member A |
| 8004247 | C17orf49 | 1.64 | 0.00062 | |
| 8088820 | RYBP | 1.63 | 0.00076 | RING1 and YY1 binding protein |
| 8058373 | WDR12 | 1.62 | 0.00077 | WD repeat domain 12 |
| 8071119 | BCL2L13 | 1.61 | 0.00092 | BCL2-like 13 (apoptosis facilitator) |
| 8174103 | GK | 1.61 | 0.00086 | glycerol kinase |
| | | | | elongation factor Tu GTP binding domain |
| 8016099 | EFTUD2 | 1.60 | 0.00079 | containing 2 |
| 7914563 | YARS | 1.60 | 0.00086 | tyrosyl-tRNA synthetase |
| 979085 | PYGL | 1.59 | 0.00092 | phosphorylase, glycogen |
| 8004237 | RNASEK | 1.59 | 0.00049 | ribonuclease, RNase K |

| 7947681 | ARHGAP1 | 1.58 | 0.00025 | Rho GTPase activating protein 1 |
|---------|-----------|-------|----------|--|
| 7959153 | COX6A1 | 1.57 | 0.00096 | cytochrome c oxidase subunit VIa polypeptide 1 |
| 8017437 | FTSJ3 | 1.56 | 0.00055 | FtsJ homolog 3 |
| | | | | eukaryotic translation initiation factor 4E family |
| 8049180 | EIF4E2 | 1.55 | 0.00096 | member |
| 8146934 | LY96 | 1.54 | 0.00075 | lymphocyte antigen 96 |
| 7900922 | ATP6V0B | 1.54 | 0.00064 | ATPase, H+ transporting |
| | | | | N-ethylmaleimide-sensitive factor attachment |
| 8037913 | NAPA | 1.53 | 0.00037 | protein, alpha |
| 8037037 | ATP5SL | 1.52 | 0.00031 | ATP5S-like |
| 8016708 | LRRC59 | 1.51 | 0.00019 | leucine rich repeat containing 59 |
| 8163383 | SUSD1 | 1.51 | 0.00062 | sushi domain containing 1 |
| 7990151 | PKM2 | 1.51 | 0.00093 | pyruvate kinase, muscle |
| 7978123 | PSME2 | 1.51 | 0.00065 | proteasome activator subunit 2 |
| 8075564 | RFPL2 | -1.51 | 0.00020 | ret finger protein-like 2 |
| 7900878 | ARTN | -1.51 | 0.00041 | artemin |
| 8141228 | TMEM130 | -1.51 | 0.00075 | transmembrane protein 130 |
| 8037298 | CD177 | -1.58 | 4.0e-005 | CD177 molecule |
| 8069142 | KRTAP10-4 | -1.59 | 0.00023 | keratin associated protein 10-4 |
| 8070771 | KRTAP10-1 | -1.60 | 0.00054 | keratin associated protein 10-1 |
| 8172713 | LOC347549 | -1.61 | 0.00061 | hypothetical LOC347549 |
| 8075200 | RHBDD3 | -1.63 | 0.00067 | rhomboid domain containing 3 |
| 8167575 | GAGE12B | -1.66 | 0.00028 | G antigen 12B |
| 8010901 | DOC2B | -1.82 | 0.00072 | double C2-like domains, beta |

Figure 1(on next page)

Percentage of peripheral blood mononuclear cells (PBMC) before and during treatment with rimonabant.

PBMC were analyzed by flow cytometry before start of therapy and 4 weeks later and results are given as percentage of mononuclear cells in blood. Each data point represents results from one patient. In cases with no change in the frequency of a certain cell type the data point would fall on the line. The only statistically significant change was for NK cells (p=0,049). For the other subsets the p-values were as follows: CD3+ p=0,47; CD3+CD4+ p=0,25; CD3+CD8+ p=0,11; CD19+ p=0,13; CD3-CD4+ p=0,11. The mononuclear gate was defined by CD45 in combination with side and forward scatter. Within this gate the frequencies of T cells (CD3+), B cells (CD19+), NK cells (CD3-, CD56+ and/or CD16+) and monocytes and dendritic cells (CD3-, CD4+) were analyzed.



Before treatment (% of mononuclear cells)