



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2570053 C 2017/01/10

(11)(21) **2 570 053**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) **Date de dépôt PCT/PCT Filing Date:** 2005/06/09
(87) **Date publication PCT/PCT Publication Date:** 2005/12/22
(45) **Date de délivrance/Issue Date:** 2017/01/10
(85) **Entrée phase nationale/National Entry:** 2006/12/11
(86) **N° demande PCT/PCT Application No.:** GB 2005/002265
(87) **N° publication PCT/PCT Publication No.:** 2005/121804
(30) **Priorité/Priority:** 2004/06/09 (GB0412859.1)

(51) **Cl.Int./Int.Cl. G01N 33/68** (2006.01),
G01N 33/569 (2006.01)
(72) **Inventeurs/Inventors:**
BRAY, DOROTHY, GB;
CLERICI, MARIO, IT;
TRABATTONI, DARIA LUCIA, IT
(73) **Propriétaire/Owner:**
IMMUNOCLIN LIMITED, GB
(74) **Agent:** FINLAYSON & SINGLEHURST

(54) **Titre : MARQUEURS POUR ATHEROSCLEROSE**
(54) **Title: MARKERS FOR ATHEROSCLEROSIS**

(57) **Abrégé/Abstract:**

A method for the diagnosis, prognosis or identification of a predisposition towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction is described, the method comprising analysing a sample to determine the percentage frequency of multiple sub-populations of T-lymphocytes and comparing the data obtained against comparative and/or control data.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
22 December 2005 (22.12.2005)

PCT

(10) International Publication Number
WO 2005/121804 A1

(51) International Patent Classification⁷: **G01N 33/68**,
33/569

(21) International Application Number:
PCT/GB2005/002265

(22) International Filing Date: 9 June 2005 (09.06.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0412859.1 9 June 2004 (09.06.2004) GB

(71) Applicant (for all designated States except US): **IM-MUNOCLIN LIMITED** [GB/GB]; Rowlandson House, 289-293 Ballards Lane, London N12 8NP (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BRAY, Dorothy** [GB/GB]; ImmunoClin Limited, Rowlandson House, 289-293 Ballards Lane, London N12 8NP (GB). **CLERICI, Mario** [IT/IT]; Milano University Medical School, DISP LITA Vialba, Via GB Grassi, 74, I-20157 Milano (IT). **TRABATTONI, Daria, Lucia** [IT/IT]; Via K. Marx 12, I-20033 Desio (IT).

(74) Agent: **MCQUEEN, Andrew, Peter**; Stevens Hewlett & Perkins, 1 St. Augustine's Place, Bristol BS1 4UD (GB).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MARKERS FOR ATHEROSCLEROSIS

(57) Abstract: A method for the diagnosis, prognosis or identification of a predisposition towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction is described, the method comprising analysing a sample to determine the percentage frequency of multiple sub-populations of T-lymphocytes and comparing the data obtained against comparative and/or control data.

WO 2005/121804 A1

MARKERS FOR ATHEROSCLEROSIS

This invention relates to markers for atherosclerosis. More particularly the present invention relates to the identification of a number of markers found in the blood and to their use in aiding the diagnosis or prognosis of, or in identifying a predisposition towards atherosclerosis and especially the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction.

Atherosclerosis is a pathological remodelling of the arteries which is a major cause of morbidity and mortality in developed countries; this pathology is the underlying basis of myocardial infarction, stroke and peripheral artery disease. Atherosclerosis can be considered as an unusual form of chronic inflammation occurring within the artery wall. A number of data support the pathogenic or pathogenetic role of inflammation in the aetiology of atherosclerosis; inflammation is an immunological phenomenon. One of the most convincing pieces of evidence for this statement stems from the observation that fatty streaks, the earliest detectable lesions in atherosclerosis, contain macrophage-derived foamy cells which are derived from circulating monocytes. T lymphocytes are also present in the atherosclerotic plaques and most of these are CD3+CD4+ alpha beta T cells. Such cells represent approximately 2/3 of all CD3+ T cells in advanced human lesions, and secrete IFN gamma, IL-2, TNF alpha and beta which cause vascular macrophage activation, vascular activation and inflammation. IFN gamma is thought to be responsible for plaque destabilization by reducing the fibrous cap. CD8+ T cells are also present in lesions but their contributory role in atherosclerosis is less clear.

The rolling, adhesion, and infiltration of immune cells (monocytes and CD4 T lymphocytes) which circulate in the peripheral blood within the endothelium is regulated by two major families of proteins expressed on the surface of immune cells, and on the surface of endothelial cells: these protein families are selectins and integrins. Chemokines or chemo-attractant cytokines are also extremely important in the control of cell trafficking and for the recruitment of immune cells at sites of inflammation. Some chemokines, those which can act as potent mediators of monocyte migration and macrophage differentiation, are expressed

in human atherosclerotic lesions. In particular, macrophage chemoattractant protein 1 (MCP-1) and its specific receptor CCR2 are strongly believed to be involved in the initial stages of atherogenesis.

Additional support for the pathogenic or pathogenetic role of inflammation in the development of atherosclerotic plaques stems from the observation made by the present inventors that different cytokines preferentially trigger the development of atherosclerotic plaques (for example, TH1-like cytokines are "bad cytokines" within this biologic scenario, that is, they trigger or are involved in the triggering of the development of atherosclerotic plaques). This becomes clearer when it is considered that TH1 cytokines are prototypic inflammatory cytokines. Cytokines and other immunological proteins present in the atherosclerotic lesions are not only produced by CD4 T lymphocytes; in fact, fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) are macrophage-derived growth factors which have been found to have significant effects on the pathology of atherosclerosis and which are macrophage-derived proteins. While the scientific knowledge of the composition of the atherosclerotic plaque and the contribution of cells found in lesions continues to increase, easy and reliable diagnosis of atherosclerosis, particularly in the absence of clinical symptoms remains a challenge as measuring and detecting changes in cell populations at the lesion site is not viable in clinical practice. Consequently there is a need for predictive and easy to assess markers that could be detected in peripheral blood samples.

The present inventors have found that the immunopathology of atherosclerotic plaques and alterations of the factors described above, and their specific receptors, result in changes of characteristics of multiple cell populations which can be measured in blood samples and in vascular biopsies of peripheral blood. Measures used may be: cell concentrations (percentages), expression of cell surface markers, intracellular concentrations of cytokines, chemokines or integrins, which alone or together with other parameters are useful in aiding the diagnosis or prognosis of, or in identifying a predisposition towards atherosclerosis and especially the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction.

Accordingly, the present invention seeks to provide a method for the diagnosis, prognosis or identification of a predisposition towards atherosclerosis which eliminates or reduces the need for invasive techniques such as the removal of a plaque for identification or for the need to use invasive optical imaging techniques.

Accordingly, the present invention provides a method for the diagnosis, prognosis or identification of a predisposition towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction, the method comprising analysing a sample to determine the percentage frequency of multiple sub-populations of T-lymphocytes and comparing the data obtained against comparative and/or control data.

As used herein, "multiple" means at least two.

Preferably, the percentages or ratios of specific cell populations are determined.

Conveniently, the sample is a blood or a biopsy sample.

In a preferred embodiment, the method comprises analysing a sample to determine the percentage frequency of one or more sub-populations of specific T-lymphocytes and comparing the data obtained against comparative and/or control data.

Preferably the method comprises determining the percentage frequency of one or more sub-populations of T-lymphocytes selected from the sub-populations (phenotypes) listed in Experiments 1 and 2 described herein.

In particular, the method comprises the determination of adhesion, activation, and rolling markers expressed on the surface of CD8+ T lymphocytes. Because these cells are the effector cells of the cellular arm of immunity, and because it is known that their activation can have a negative impact on disease development via destruction of target cells (e.g. the destruction of liver cell that are infected by hepatitis viruses is mediated by CD8 T cells and is associated with worsening of the disease) or via the induction of a chronic inflammatory condition, the inventors believe that focussing on these cells will allow a more accurate determination of the predisposition towards

atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction,

Additionally, the method comprises determining the simultaneous percentage frequency of multiple sub-populations of lymphocytes. This proposal stems from the fact that the predisposition towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction is multi-factorial and thus it will be impossible to accurately predict such predisposition with the analysis of one single marker.

Thus, in one embodiment the method comprises determining the percentage frequency of multiple sub-populations of T-lymphocytes wherein at least one of said sub-populations is a sub-population of CD8⁺ T-lymphocytes selected from the sub-populations (phenotypes) of CD8⁺/CD11L⁺/CD45RA⁺, CD8⁺/CD11L⁺/CD45RO⁺, CD8⁺CD11a⁺, CD8⁺CD62L⁺ and CD8⁺CD38⁺RO⁺.

In another embodiment, the method comprises determining the percentage frequency of multiple sub-populations of T-lymphocytes wherein at least one of said sub-populations is a sub-population of CD4⁺ T lymphocytes selected from the following phenotypes: CD4⁺CD25⁺, CD4⁺DRII⁺, CD4⁺CD44⁺, CD4⁺CD62L⁺ and CD4⁺CD49d⁺.

In a preferred embodiment, the method comprises determining the percentage frequency of multiple sub-populations of T-lymphocytes wherein at least one of said sub-populations is a sub-population of CD8⁺ T-lymphocytes and at least one other of said sub-populations is a sub-population of CD4⁺ T-lymphocytes. Preferably the CD8⁺ and CD4⁺ sub populations are selected from the phenotypes listed above.

Particularly preferred CD8⁺ sub-populations are: CD8⁺/CD11L⁺/CD45RA⁺ and CD8⁺/CD11L⁺/CD45RO⁺. Particularly preferred CD4⁺ sub-populations are CD4⁺CD62L⁺ and CD4⁺CD49d⁺. Ideally, one or more of each of the foregoing CD8⁺ and CD4⁺ sub-populations are measured together.

The biological reasons that led to the definition of such panel are detailed as follows:

- CD8/CD11L/CD45RA; post-naive cells that express CD11 (aka LFA-1alpha) with a low expression density, thus these are cells that express low levels of the adhesion CD11; this low expression makes them stick less to the endothelium. The reduction of these cells in atherosclerosis means that there are fewer circulating cells with a low "sticking ability" to the endothelium. These cells are similar to central memory cells and migrate to periphery as a likely effect of a 'negative feedback' resulting from plaque formation.
- CD8+/CD11L+/CD45RO+; these are CD8 T cells that are augmented in inflammation, similar to the phenotype of effector memory cells; these cells also have reduced ability of sticking to epithelium and may be a product of 'negative feedback' as a result of plaque formation.
- CD4/CD62L; CD62L is an adhesion (aka L selectin) thus an adhesion marker expressed on CD4 and utilized by these cells to adhere to the high endothelial venules (HEV) and penetrate within the vascular wall.
- CD4/CD25 and CD8/DRII; CD25 is the receptor for IL-2 and its expression is correlated with inflammation. DRII is expressed by CD4 and CD8 upon activation, thus the inventors' data confirms the inflammatory status associated with atherosclerosis.
- CD4/CD44 and CD4/CD49d; (cd44 aka M-CAM; CD49d aka VLA-4alpha); CD44 and CD49d are adhesion molecules; in theory they should increase in atherosclerosis; the decrease could be secondary to a negative feed-back (i.e. a way in which the body tries to stop the atherogenetic process).

The predictive value of the method of the invention may be augmented by supplementing the analysis of different lymphocyte sub-populations with further tests for other immunologic markers implicated in the development of atherosclerotic plaques and vascular obstruction. The use of multiple markers facilitates prediction of disease progression with better predictive value and, hence, provides a better outcome for the patient because appropriate treatment can be initiated earlier. Multiple immune correlates provide a powerful predictive tool because there is interdependence between the markers in contrast to routine diagnostics where very few results depend upon each other.

Accordingly, in a further embodiment, the invention provides a method for the diagnosis, prognosis or identification of a predisposition towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction as described above, which further comprises determining the concentration of one or more soluble factors intracellularly, in plasma or in serum and comparing the data obtained against comparative and control data. Preferably, said soluble factor is selected from the group consisting of cytokines and chemokines. Most preferably, the cytokines are activation cytokines.

In a still further embodiment, the invention provides a method for the diagnosis, prognosis or identification of a predisposition towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction as described above, which further comprises one or more of additional steps (a) and (b) :

- (a) determining the percentage frequency of cells expressing activation cytokines, and/or chemokines, and/or integrins, and/or selectins in the sample;
- (b) determining the frequency of cell surface molecules responsible for cell activation

and comparing the data obtained against comparative and control data.

It is preferred that the all of the above information is obtained from the same blood sample, and in addition, the chemokine and cytokine data may be obtained from serum or plasma. Additionally, the information may be obtained from a biopsy sample. However, it is preferred that the method is used with a blood sample in order to avoid or reduce the need for taking biopsy samples.

The preferred cytokines include interleukin-1 (α or β), interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin-12, interleukin-13, interleukin-14, interleukin-15, interleukin-16, interleukin-17, interferon- α , interferon- β , interferon- γ , tumour necrosis factors such as TNF- α and TNF- β , fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), Colony stimulating factors such as G-CSF, GM-CSF, M-CSF, and transforming growth

factors such as TGF- β . Of these, TNF- α , IL-12, interferon- α , FGF, and PDGF are especially preferred.

The preferred chemokines include CC and CXC chemokines.

The method may include the additional step of determination of the concentration of macrophage chemoattractant protein 1 (MCP-1) in a sample from the same patient.

Optionally, the method may also comprise the mathematical steps of determining the statistical changes in immunological parameters which may include lymphocyte population ratios; for example the ratio of CD8+/CD11L+/CD45RA+ lymphocytes to CD8+/CD11L+/CD45RO+ lymphocytes, as these two populations are likely to be a part of the lymphocyte maturation pathway and the ratios may therefore help to determine whether the maturation process is altered.

Embodiments of the invention will now be described purely by way of non-limiting example in which reference is made to the figures of which:-

Figure 1 a to f. Describes changes in lymphocyte subpopulations in peripheral blood of HIV infected individuals who did (pl+) or did not (pl-) develop atherosclerotic plaques (carotid artery) as determined by echo doppler evaluations and in healthy controls.

Figure 2 a to h. Describes changes in T cell populations in peripheral blood of HIV negative individuals who did (pl+) or did not (pl-) develop atherosclerotic plaques (carotid artery) as determined by echo doppler evaluations.

Experiment 1: T cell populations were assessed in peripheral blood of HIV infected individuals who did (pl+) or did not (pl-) develop atherosclerotic plaques (carotid artery) as determined by echo doppler evaluations. Data from 10 sex and age matched healthy controls were also obtained. The data are presented in Tables I and II and in Figure 1.

Table I. Analysis of a number of different lymphocyte subpopulations by FACS methods in peripheral blood of HIV infected individuals who did (pl+) or did not

(pl-) develop atherosclerotic plaques (carotid artery) as determined by echo doppler evaluations in Experiment 1.

Phenotype	t-test	Mean	
	pl+ vs pl -	pl+	pl-
CD8+CD25+	0,374225892	2,36	5,80
CD4+CD25+	0,072409562	2,91	0,56
CD8+DRII+	0,282417879	19,83	7,11
CD4+DRII+	0,030600648	23,09	1,55
CD4+CD44+	0,00395716	70,60	20,36
CD8+CD44+	0,160297549	72,80	51,30
CD4+CD11a+	0,225448719	51,05	29,99
CD8+CD11a+	0,074474538	87,70	52,83
CD4+CD62L+	0,21745848	35,67	13,27
CD8+CD62L+	0,029642338	41,87	20,42
CD8+CD38+RO+	0,00295714	12,80	2,35
CD8+CD28-RA+	0,245463793	36,17	52,72
CD4+CD3+RO+	0,085958624	69,50	41,58
CD4+CD62L+RA+	0,654853858	20,98	18,12
CD8+CD11highRA+	0,166981489	13,57	14,80
CD8+CD11lowRA+	0,344892909	4,93	16,08
CD8+CD11highRO+	0,044961021	28,00	10,80
CD4+CD49d	0,034261111	39,10	12,98
CD8+CD49d	ND	40,10	47,80

The patients (N=15/group) were comparable in vitro-immunologic terms and had been undergoing a PI-based ARV.

Table II. Data obtained in 10 sex and age matched healthy controls in Experiment 1.

Phenotype	Mean	sd	Se
CD8+CD25+	2,14	0,17591935	0,07
CD4+CD25+	0,84	0,22154221	0,09
CD8+DRII+	7,22	4,19724456	1,71
CD4+DRII+	1,77	0,9772532	0,4
CD4+CD44+	42,16	5,68561259	2,32
CD8+CD44+	29,21	3,33288092	1,36
CD4+CD11a+	19,60	5,92143001	2,42

CD8+CD11a+	30,93	3,40377941	1,39
CD4+CD62L+	32,19	5,790345	2,36
CD8+CD62L+	20,67	3,95462357	1,61
CD8+CD38+RO+	3,83	2,68271451	1,09
CD8+CD28-RA+	49,40	8,18005705	3,34
CD4+CD3+RO+	45,53	5,96984486	2,44
CD4+CD62L+RA+	18,64	5,45858869	2,23
CD8+CD11highRA+	18,10	5,76136558	2,35
CD8+CD11lowRA+	4,41	3,05692033	1,25
CD8+CD11highRO+	15,23	5,00723286	2,04
CD4+CD49d	9,57	2,21578802	
CD8+CD49d	NT		

Experiment 2: T cell populations were assessed in peripheral blood of HIV negative individuals who did (pl+) or did not (pl-) develop atherosclerotic plaques (carotid artery) as determined by echo doppler evaluations. The data are presented in Table III and Figure 2.

The present inventors' initial observations were made on HIV infected patients. Because HIV is an immune deficiency, it is possible that the differences in lymphocyte populations observed in HIV infected patients may not be exactly mirrored in individuals not infected with HIV. Accordingly, the inventors studied lymphocyte populations in patients not having any other disease but with a positive diagnosis of thickening of the intima as determined by echo Doppler and in healthy controls as confirmed by echo Doppler.

When these data were compared with those for HIV infected patients, it was observed that several of the lymphocyte population markers were altered in the same direction (*i.e.* increased or decreased frequency in patients who developed plaques versus patients who did not) in the non-HIV infected patients as had previously been observed in the HIV infected patients. The HIV + patients in experiment 1 were in advanced stage of HIV disease and were treated with protease inhibitors which were to be associated with a statistically significant increase in the risk of cardiovascular disease. HIV disease is an immunodeficiency and dramatic alteration in lymphocyte markers is expected to

be progressively greater with advanced disease. The uniformity of changes in multiple populations of lymphocytes in atherosclerotic patients both experiments is surprising in the view of the expected discrepancies between immunodeficient HIV infected population and non HIV infected patients. Accordingly, these data confirm the utility of changes in these lymphocyte populations as markers for atherosclerosis.

There were some discrepancies between the results of Experiment 1 (HIV infected patients) and Experiment 2 (non-HIV infected), but this was in lymphocyte populations known to be affected by HIV.

Lymphocyte populations which were observed to be altered in the same direction for patients who developed plaques in both the HIV infected and non-infected patient studies are the most promising diagnostic markers for atherosclerosis. However, it might be argued that lymphocyte populations which were observed to alter in opposite directions in the two experiments might still be useful as markers since an observed change of this population in a patient in either direction could be an indication that further investigation is warranted.

Interestingly, monocytic populations expressing CD86 and CD80 were found to be decreased in patients with atherosclerosis. These decreases may be a reflection of monocyte migration into atherosclerotic plaques.

Table III a and b shows phenotypic analysis of a number of different lymphocyte subpopulations in peripheral blood of individuals who developed atherosclerotic plaques (ATHERO1) and healthy controls (HC) in part a and levels of plasmatic cytokines in part b. It is of note that as expected levels of MCP-1 were higher in atherosclerosis patients as compared to healthy controls.

Table III a: Phenotypic Analysis (% of positive cells)

	patient	CD4	CD8	CD4/CD25	CD8/CD25	CD4/CD62L	CD4/DRII	CD8/DRII	CD4/CD44	CD8/CD44	CD4/CD49d	CD4/CD11a
ATHERO	1	11.5	45	0.45	0.62	6.2	0.54	6.12	10.3	45.3	7.28	10.9
	2	13.5	47	4.38	14.6	8.24	1.2	10.7	14.9	46.9	3.69	5.59
	3	23.2	48.3	0.57	0.83	6.7	0.66	7.34	21.1	21.8	5.18	16.9
	4	48.2	23.5	0.56	0.1	25.2	0.44	12.9	46.6	18	4.56	
	5	51.9	18.7	0.19	0.08	37.3	0.66	9.08	45.2	19.9	3.23	13.4
	6	57.2	14	0.37	0.12		1.26	1.22	42.3	11.6		20.6
	7	44.3	16.2	0.79	0.1	32.4	0.48	1.26	39.7	17.2		16.9
	8	39.5	15.4	0.35	0.05	24.9	1.58	0.3	22	16.5	2.97	20.9
	9	40	12.5	0.56	0.09	5.96	1.44	2	39.3	13	23.6	31.3
	10	41.9	21.8	0.35	0.02	27.2	1.3	1.58	41.9	18.9	13.4	21.8
HC	1	58.3	16.2	1.25	0.55		0.7	0.68	57.6	21.7	46.8	9.47
	2	46.3	14.7	1.17	0.47	4.76	0.62	2.74	49.2	23.5	39.8	9.82
	3	48.8	15.2	0.56	0.32	4.56	0.44	0.22				20.3
	4	43.2	22.2	1.62	0.53	5.62	0.6	1.44	45.9	26.8		22.6
	5	40.8	18.3	0.6	2.17	2.9	0.44	2.98	42.1	27.1	25	13.3
ATHERO	mean	37.12	26.24	0.86	1.66	19.34	0.96	5.25	32.33	22.91	7.99	17.59
	median	40.95	20.25	0.51	0.10	24.90	0.93	4.06	39.50	18.45	4.87	16.90
	sd	15.78	14.57	1.25	4.55	12.52	0.44	4.58	13.68	12.59	7.17	7.36
	se	5.26	4.86	0.42	1.52	4.17	0.15	1.53	4.56	4.20	2.39	2.45
	mean	47.48	17.32	1.04	0.81	4.46	0.56	1.61	48.70	24.78	37.20	15.10
	median	46.30	16.20	1.17	0.53	4.66	0.60	1.44	47.55	25.15	39.80	13.30
	sd	6.77	3.06	0.45	0.77	1.14	0.12	1.22	6.60	2.62	11.13	6.04
	se	3.38	1.53	0.23	0.38	0.57	0.06	0.61	3.30	1.31	5.57	3.02
HC	mean	37.12	26.24	0.86	1.66	19.34	0.96	5.25	32.33	22.91	7.99	17.59
	median	40.95	20.25	0.51	0.10	24.90	0.93	4.06	39.50	18.45	4.87	16.90
	sd	15.78	14.57	1.25	4.55	12.52	0.44	4.58	13.68	12.59	7.17	7.36
	se	5.26	4.86	0.42	1.52	4.17	0.15	1.53	4.56	4.20	2.39	2.45

t-test		CD4	CD8	CD4/CD25	CD8/CD25	CD4/CD62L	CD4/DRII	CD8/DRII	CD4/CD44	CD8/CD44	CD4/CD49d	CD4/CD11a
ATHERO vs HC		0.189306996	0.206247327	0.759599836	0.689634442	0.040899724	0.073187783	0.109820352	0.044050829	0.779001962	0.000525575	0.53258279

	patient	CD8/CD11a	CD8/CD11a/CD45RA	CD8/CD11/CD45RA	CD8/CD11/CD45RO	CD14/CD86	CD14/CD80	CD14/TLR2	CD14/TLR4	CD14/CD36
ATHERO1	1					33.7	72	99	16.7	83.5
	2	42.5	18.7	2.14	79.12	85.8	80.6	98.4	33.3	99.7
	3	45	7.44	0.55	92.02	78.1	87.8	99.2	41.9	99.8
	4	18.4	25.5	5.52	67.32	85.5	76.6	99.7	85	98.9
	5	12.3	15.3	12.8	71.9	78.5	73.1	99.6	75.9	98.6
	6	10.1	24.6	21.4	54	83.8	63.3	100	41.8	96.7
	7	8.55	25.6	19.8	54.6	69.9	37.2	100	72.6	86.5
	8	13.9	29.5	34.9	33.72	90	57.8	99.7	49.8	95.1
	9	6.57	13.8	38.1	48.1	63.4	43.4	99.4	72	89.4
	10	22.5	37.5	11.4		63	59.3	99.6	2.75	97.3
HC	1	11.2	25.5	34.6	39.97	85.1	73.9	99.9	3.45	97.8
	2	8.84	39.3	27.3	31.03	92.2	83	100	56	96.5
	3	6.15	33.3	25.5	41.23	97	87.2	99.6	55.7	99.2
	4	14.4	15.8	34.1	50.11	86.7	78.2	99.6	21.3	97.7
	5	11.7	16.9	42.8	40.24	89.4	84.9	100	34.6	98.7

	CD8/CD11a	CD8/CD11a/CD45RA	CD8/CD11/CD45RA	CD8/CD11/CD45RO	CD14/CD86	CD14/CD80	CD14/TLR2	CD14/TLR4	CD14/CD36
ATHERO	mean	19.98	21.99	16.29	73.17	65.11	99.46	49.18	94.55
	median	13.90	24.60	12.80	78.30	67.65	99.60	45.85	97.00
	sd	14.34	9.11	13.51	16.76	16.12	0.49	27.16	5.92
	se	4.78	3.04	4.50	5.59	5.37	0.16	9.05	1.97
HC	mean	10.46	26.16	32.86	90.08	81.44	99.82	34.21	97.98
	median	11.20	25.50	34.10	89.40	83.00	99.90	34.60	97.80
	sd	3.11	10.21	6.86	4.72	5.36	0.20	22.64	1.04
	se	1.56	5.11	3.43	2.36	2.68	0.10	11.32	0.52

t-test	CD8/CD11a	CD8/CD11a/CD45RA	CD8/CD11/CD45RA	CD8/CD11/CD45RO	CD14/CD86	CD14/CD80	CD14/TLR2	CD14/TLR4	CD14/CD36
ATHERO vs H	0.175241575	0.446437511	0.02621318	0.028811029	0.048593792	0.049118744	0.143107564	0.309772197	0.228797111

Table IIIb:Plasmatic Cytokines (pg/ml)

patient		s-ICAM-1	s-VCAM-1	RANTES	MIP-1 alpha	MIP-1 beta	MCP-1	IL-1	IFN-alfa	TNF-alfa
ATHERO	1	29.7	43.2	2498.5	4.8	20.2	83.6	0	0	0
	2	28.5	38.8	3304.8	8.9	123.6	186.0	1	17.2	7.5
	3	27.8	50.7	3321.2	16.0	52.2	110.8	0	50	1.3
	4	20.9	31.7	3272.0	5.9	73.8	158.0	0	0	0
	5	13.9	35.4	3274.2	6.5	68.2	65.0	0	2000	0
	6	32.7	31.7	3432.2	8.9	53.6	92.5	0	6.7	0
	7	16.7	29.7	3450.8	12.5	101.8	51.9	0	6.5	0
	8	17.9	32.5	3263.8	1.8	24.3	152.4	0	12.5	10.7
	9	21.1	25.6	2903.1	6.5	32.3	104.4	0	12.5	0
	10	19.1	47.8	2876.3	1.8	34.0	64.5	0	6.2	0
HC	1	23.1	33.9	3255.6	2.4	91.6	99.1	0	6.3	0
	2	14.9	29.8	3357.7	6.5	46.3	78.8	0.1	6.2	0
	3	15.9	32.1	3281.7	5.3	49.9	60.0	0	6.2	0
	4	11.6	25.7	3304.8	1.2	69.9	39.3	0	41.7	0
	5	20.0	37.7	3368.9	46.3	26.4	2.8	0	228.1	0

		s-ICAM-1	s-VCAM-1	RANTES	MIP-1 alpha	MIP-1 beta	MCP-1	IL-1	IFN-alfa	TNF-alfa
ATHERO	mean	22.83	36.71	3159.69	7.36	58.39	106.92	0.10	211.16	1.95
	median	20.99	33.96	3273.10	6.53	52.86	98.45	0.00	9.60	0.00
	sd	6.35	8.22	302.95	4.45	34.04	45.07	0.30	628.70	3.87
	se	2.12	2.74	100.98	1.48	11.35	15.02	0.10	209.57	1.29
HC	mean	17.09	31.83	3313.74	12.35	56.82	55.99	0.02	57.70	0.00
	median	15.87	32.10	3304.80	5.34	49.92	60.02	0.00	6.30	0.00
	sd	4.49	4.49	48.64	19.11	24.83	37.08	0.04	96.50	0.00
	se	2.25	2.24	24.32	9.55	12.42	18.54	0.02	48.25	0.00

t-test	s-ICAM-1	s-VCAM-1	RANTES	MIP-1 alpha	MIP-1 beta	MCP-1	IL-1	IFN-alfa	TNF-alfa
ATHERO vs HC	0.096237187	0.2425894	0.28734583	0.431848851	0.92870151	0.04879082	0.5948942	0.60314087	0.28809286

Table IV. Lymphocyte markers cited in Experiments 1 and 2

Marker	Description	Experiment 1 In HIV+ pts PI+ vs. pl-	Experiment 2 In HIV neg pts PI+ vs. PL-
CD8+CD25+	CD25 is the receptor for IL-2 and its expression is correlated with inflammation. CD25-expressing lymphocytes are activated lymphocytes (an indirect index of inflammation)	Decrease in pl+ when compared to HIV+ but not when compared to healthy controls	Increase in pl+
CD4+CD25+	CD25 is the receptor for IL-2 and its expression is correlated with inflammation. CD25-expressing lymphocytes are activated lymphocytes (an indirect index of inflammation)	Increase in pl+	No change in PI+
CD8+DRII+	DRII is expressed by CD4 and CD8 upon activation. DRII-expressing lymphocytes: same value as CD25	Increase in pl+	Increase in pl+
CD4+DRII+	DRII is expressed by CD4 and CD8 upon activation. DRII-expressing lymphocytes: same value as CD25	Increase in pl+	Increase in pl+
CD4+CD44+	CD44 (M-CAM) is an adhesion molecule: circulating lymphocytes stick to the endothelial cells; this facilitates their penetration in the endothelium	Increase in pl+	Decrease in PI+
CD8+CD44+	CD44 (M-CAM) is an adhesion molecule: circulating lymphocytes stick to the endothelial cells; this facilitates their penetration in the endothelium	Increase in pl+	No change in pl+
CD4+CD11a+	CD11 a is AlphaL integrin chain important for intercellular adhesions, and costimulatory signaling	Increase in pl+	Increase in pl+
CD8+CD11a+	CD11 a is AlphaL integrin chain important for intercellular adhesions, and costimulatory signaling	Increase in pl+	Increase in pl+
CD4+CD62L+	CD62L is an adhesin (L selectin). An adhesion marker expressed on CD4 and utilized by these cells to adhere to the high endothelial venules (HEV) and penetrate within the vascular wall.	Increase in pl+	Increase in pl+

CD8+CD62L+	CD62L is an adhesin (L selectin). An adhesion marker expressed on CD8 and utilized by these cells to adhere to the high endothelial venules (HEV) and penetrate within the vascular wall.	Increase in pl+ patients	Not done
CD8+CD38+CD45RO+	memory-activated T lymphocytes: an indirect index of inflammation and immune activation	Increase in pl+ patients	Not done
CD8+CD28-CD45RA+	T suppressor, IL-10-producing cells: the lower their value the worst the inflammatory condition	No change	Not done
CD4+CD3+CD45RO+	memory-activated T lymphocytes: an indirect index of inflammation and immune activation	Increase in pl+	Not done
CD4+CD62L+CD45RA+	naive T lymphocytes	No change	Not done
CD8+CD11high+CD45RA+	CD11 is an adhesion molecule: circulating lymphocytes stick to the endothelial cells; this facilitates their penetration in the endothelium	No change	Not done
CD8+CD11low+CD45RA+	CD11 is an adhesion molecule. Cells that express low levels of the adhesin CD11 makes them stick less to the endothelium..	Decrease in PI+	Decrease in PI+
CD8+CD11a+CD45RA+	CD11 a is AlphaL integrin chain important for intercellular adhesions, and costimulatory signaling	Not done	No change
CD8+CD11high+CD45RO+	CD11 is an adhesion molecule: circulating lymphocytes stick to the endothelial cells; this facilitates their penetration in the endothelium	Increase in PI+ patients	Not done
CD8+CD11low+CD45RO+	CD11 is an adhesion molecule; these are CD8 T cells that augment in inflammation.	Not done	Increase in pl+
CD4+CD49d+	CD49 (VLA-4 alpha) is an adhesion molecule: circulating lymphocytes stick to the endothelial cells; this facilitates their penetration in the endothelium	Increase in PI+ patients	Decrease in PI+
CD8+CD49d+	CD49 (VLA-a alpha) is an adhesion molecule: circulating lymphocytes stick to the endothelial cells; this facilitates their penetration in the endothelium	No change	Not done
CD14+CD86+	CD86 is also known as B7.2 Binds	Not done	Decrease in PI+

	Cd28 responsible for co-stimulation of T cells, is thought to be mostly TH2		
CD14 +CD80+	CD80 is also known as B7.1 ; it is thought to be mostly Th1	Not done	Decrease in PI+
CD14 +TLR2+	TLR2= TLR receptor	Not done	No change
CD14 + TLR4+	TL4=TLR receptor	Not done	No change
CD14 + CD36+	CD36 is activation marker	Not done	No change

Preferably, the method includes the further step of assessing the thickness of the vascular intima to assess if there is thickening which may be indicative of the presence or likelihood of atherosclerosis or atherosclerotic plaques. In order to achieve this it is preferred that other non-invasive techniques such as ultrasonography, including Doppler ultrasound techniques, are used in conjunction with the method of the present invention. Further, the present invention may be used as a replacement for or in addition to conventional invasive imaging techniques.

The inventors have shown that alterations in the parameters described above, in particular, changes in T cell populations such as CD8+/CD11L+/CD45RA+, CD8+/CD11L+/CD45RO+, CD8+CD11a+, CD8+CD62L+, CD8+CD38+RO+, assessed together with CD4+CD3+RO+, CD8+CD11highRO+, CD4+CD62L+, CD4+CD49d+, CD4+CD25+, CD4+DRII+, CD4+CD44+, are indicative of plaque formation. Where analysis according to the invention indicates a high risk that a plaque has formed or is imminent, this may be confirmed by invasive test. Advantageously, the test according to the invention is performed first at regular intervals and the invasive test only performed if the raised frequency for one or more of the indicator cell populations is observed.

It is envisaged that data from the method of the invention will be interpreted using computer software and graphic representation.

Methods used to measure cells include: labelling of whole blood in a single tube; lysing red blood cells; and reading using flow cytometry. Alternatively,

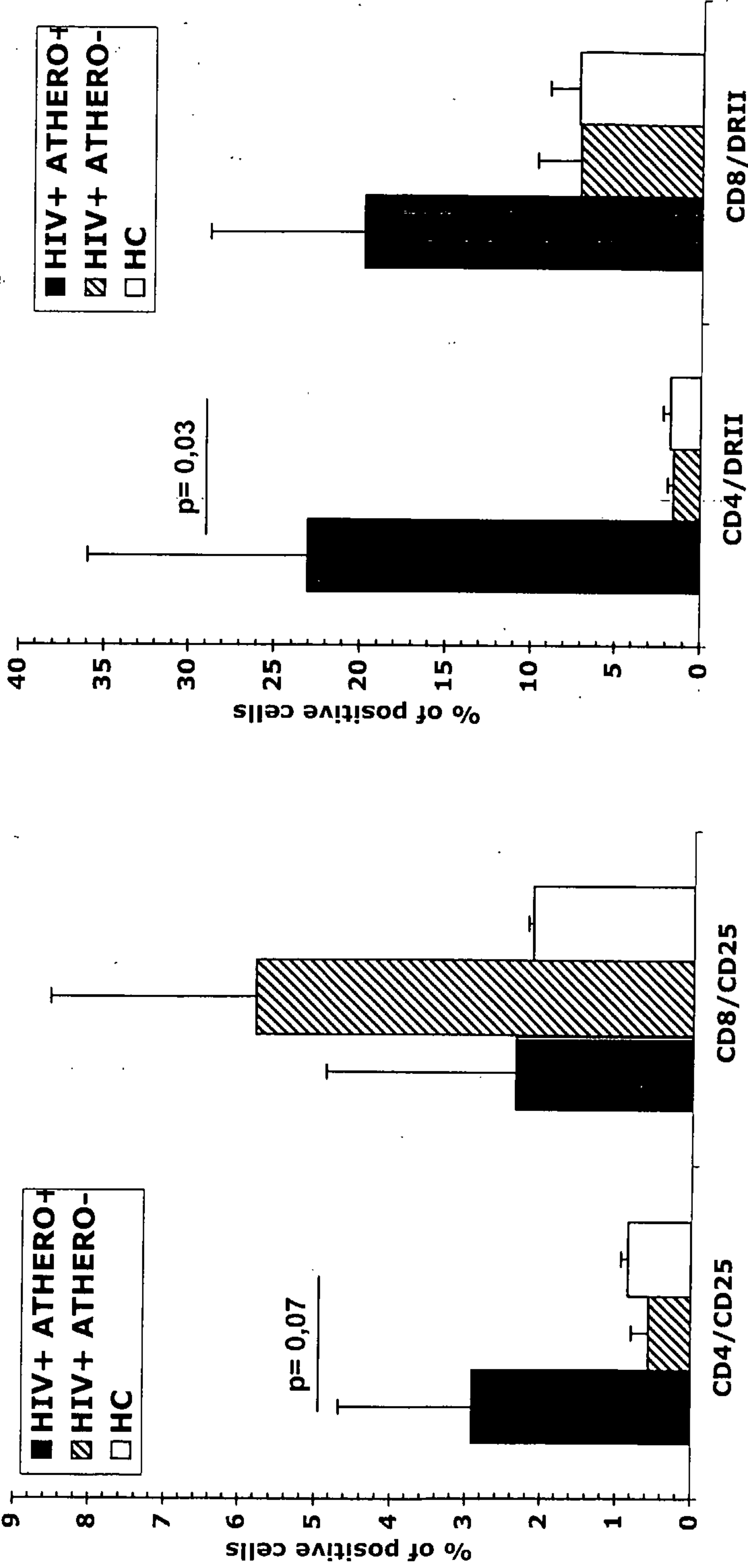
labelled cells may be added to a solid phase whereby cells are counted using an image analyzer and customized software is used to provide automated output of data: output may be tailored to compare single patients' results to a database of results from healthy individuals and to a database of data from varying stages of the disease to facilitate accurate prediction of plaque formation.

CLAIMS

1. A method for the diagnosis, prognosis or identification of a predisposition of a peripheral blood sample donor towards atherosclerosis and the susceptibility to the development of at least one of atherosclerotic plaques and vascular obstruction, the method comprising analysing the donor's peripheral blood sample to determine the percentage frequency of at least two sub-populations of T-lymphocytes in the peripheral blood sample and comparing the data against comparative and/or control data, wherein at least one of said sub-populations of T-lymphocytes is a CD8 T cell population selected from the list consisting of CD8+/CD11L+/CD45RA+, CD8+/CD11L+/CD45RO+, CD8+CD11a+, CD8+CD62L+, CD8+CD11highCD45RO+, or a CD4 T cell population selected from the list consisting of CD4+/62L+, CD4+CD49d+, CD4+CD25+, CD4+HLADRII+, CD4+CD44+ and CD4+CD3+CD45RO+.
2. A method for the diagnosis, prognosis or identification of a predisposition of a peripheral blood sample donor towards atherosclerosis and the susceptibility to the development of atherosclerotic plaques and/or vascular obstruction, the method comprising analysing the donor's peripheral blood sample to determine the percentage frequency of at least two sub-populations of T-lymphocytes in the peripheral blood sample and comparing the data against comparative and/or control data, wherein at least one of the said subpopulations of cell population is a CD8 T cell population selected from the list consisting of CD8+/CD11L+/CD45RA+, CD8+/CD11L+/CD45RO+, CD8+CD11a+, CD8+CD62L+, CD8+CD11highCD45RO+, or a CD4 T cell population selected from the list consisting of CD4+/62L+, CD4+CD49d+, CD4+CD25+, CD4+HLADRII+, CD4+CD44+ and CD4+CD3+CD45RO+, and a monocyte population selected from the list consisting of CD14+CD86+ and CD14+CD80+.
3. A method according to any one of claims 1 to 2, wherein the sample is a blood or a biopsy sample.
4. A method according to any one of claims 1-3 which further comprises determining the concentration of one or more soluble factors intracellularly, in plasma or in serum and comparing the data obtained against comparative and control data.

5. A method according to claim 4, wherein said soluble factor is selected from the group consisting of cytokines and chemokines.
6. A method according to any one of claims 1-5 which further comprises one or more of additional steps (a) and (b):
 - (a) determining the percentage frequency of cells expressing activation cytokines, and/or chemokines, and/or integrins, and/or selectins in the sample;
 - (b) determining the frequency of cell surface molecules responsible for cell activation and comparing the data obtained against comparative and control data.
7. A method according to claim 5 or 6, in which the cytokine is one or more of interleukin-1 (α or β), interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin-12, interleukin-13, interleukin-14, interleukin-15, interleukin-16, interleukin-17, interferon- α , interferon- β , interferon- γ , tumour necrosis factors TNF- α and TNF- β , fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), Colony stimulating factors G-CSF, GM-CSF, and MLSF, and transforming growth factors TGF- β .
8. A method according to claim 7, in which the cytokines are TNF- α , IL-12, interferon- α , FGF, and PDGF.
9. A method according to claim 6 or 7, in which the chemokines include CC and CXC chemokines.
10. A method according to any one of claims 1-9, in which the method further comprises the additional step of determination of the concentration of macrophage chemoattractant protein 1 (MCP-1) in the sample from the same patient.

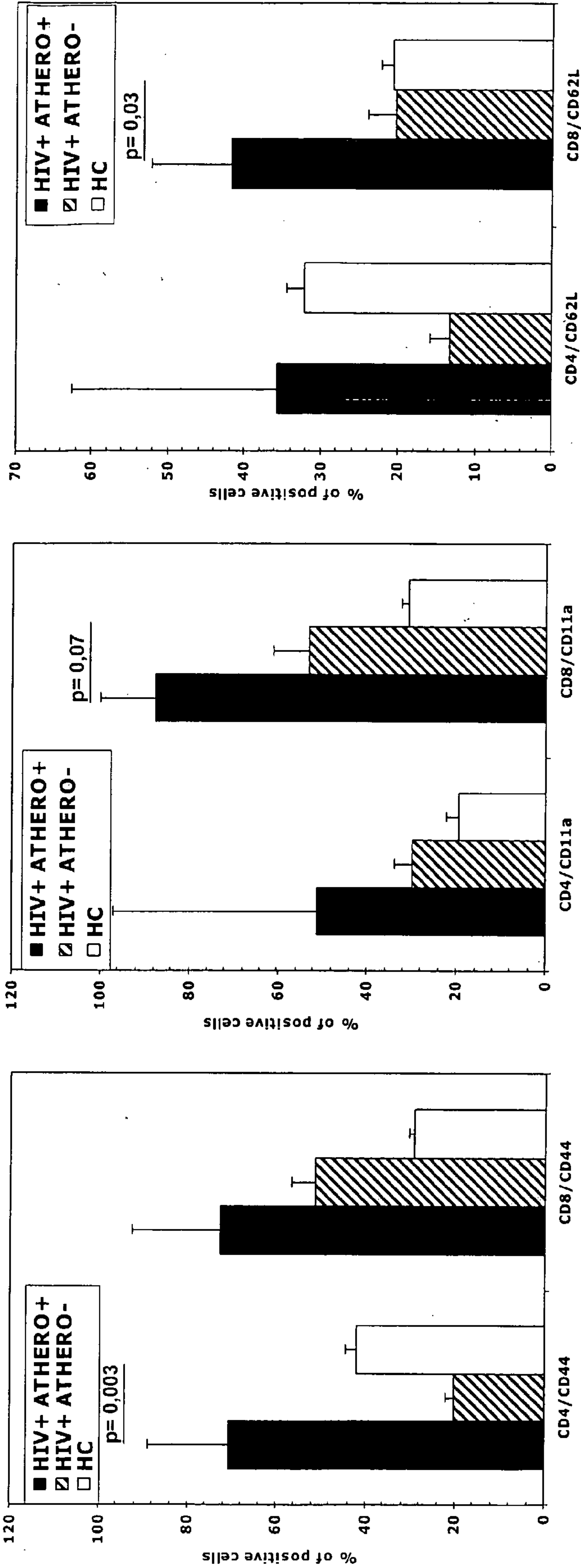
11. A method according to any one of claims 1-10, in which the method further comprises the additional step of determining the statistical changes in immunological parameters.
12. A method according to claim 11, in which the immunological parameter is the lymphocyte ratios.
13. A method according to any one of claims 1-12, in which the method further comprises the step of assessing the thickness of the vascular intima to determine if there is thickening which may be indicative of the presence or likelihood of atherosclerosis or atherosclerotic plaques.
14. A method according to claim 13, in which the assessment of the thickness of the vascular intima is by ultrasonography.
15. A method according to any one of claims 1-14, in which the data is interpreted using computer software and graphic imaging.
16. A method according to claim 1 or 2, in which analysis of the blood sample comprises labelling of whole blood in a single tube.
17. A method according to claim 16, in which analysis of the blood sample comprises the further steps of lysing red blood cells, and analyzing using flow cytometry.
18. A method according to claim 17, in which analysis of the blood sample comprises the further steps of adding the labelled cells to a solid phase, and using an image analyzer to count cells.
19. A method according to claim 17 or 18, in which computer software is used to compare single patient's results to a database of healthy individual's results and to a database of data from varying stages of the disease to facilitate accurate prediction of the plaque formation.



mean values + SE

Fig. 1a

Fig. 1b



mean values $\pm$ SE

3/14

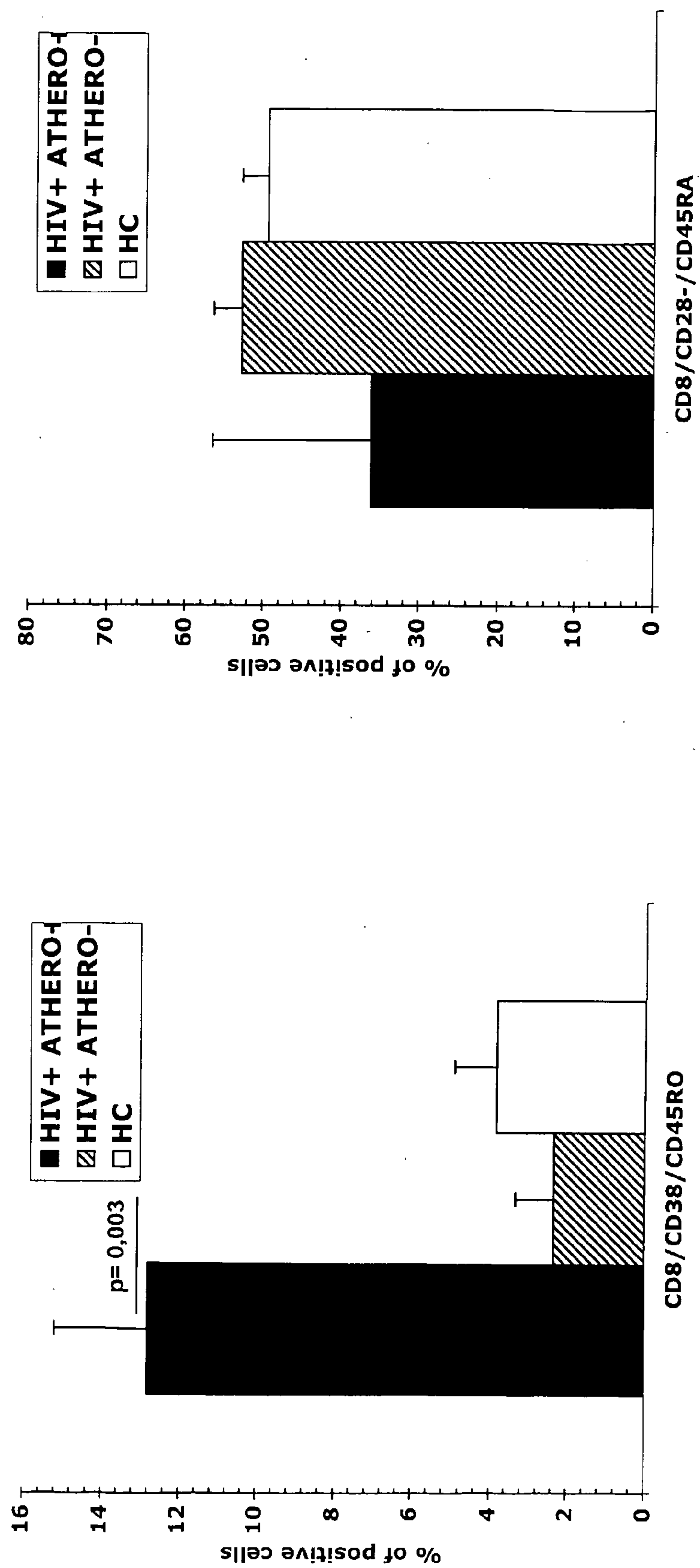
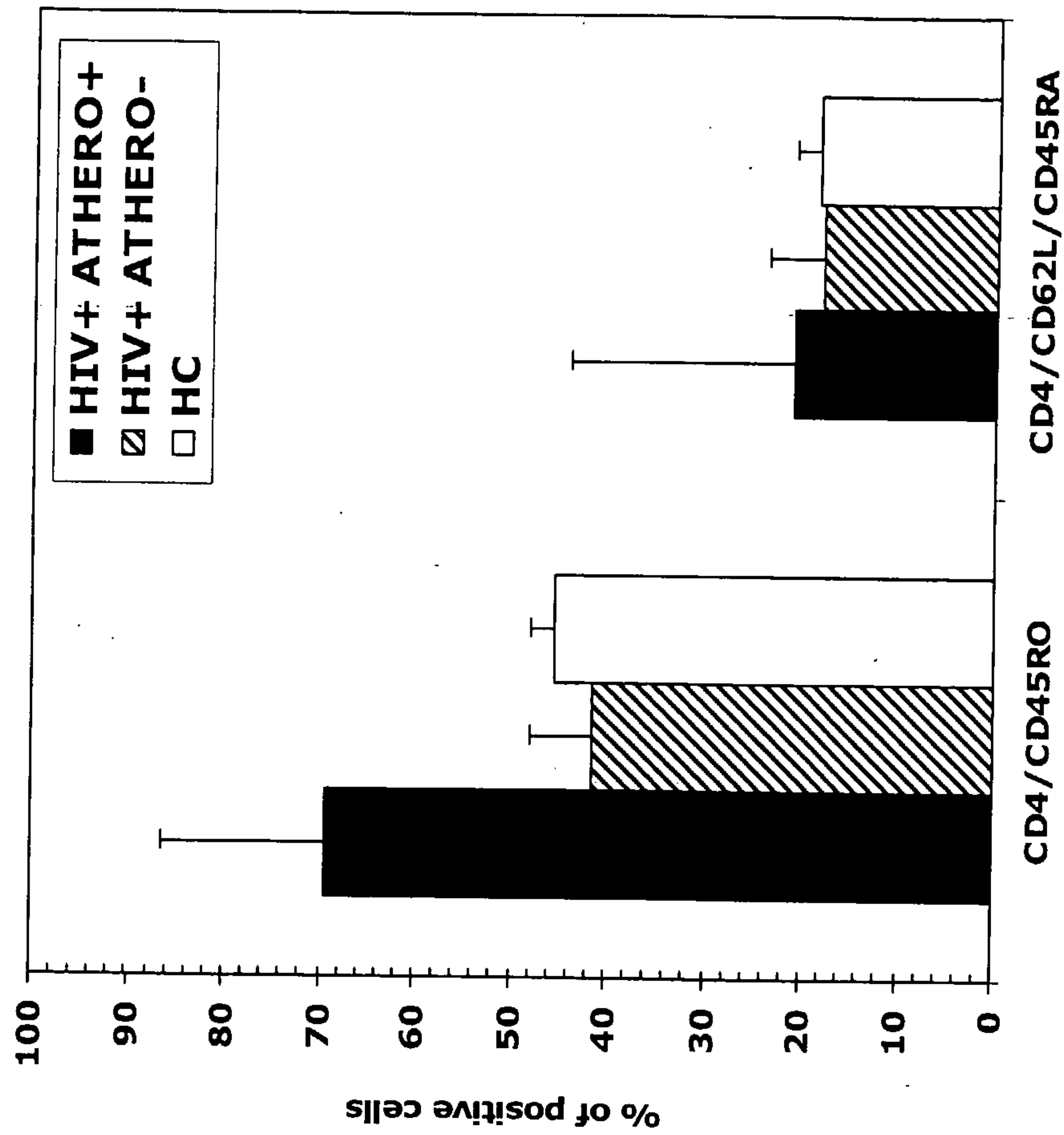
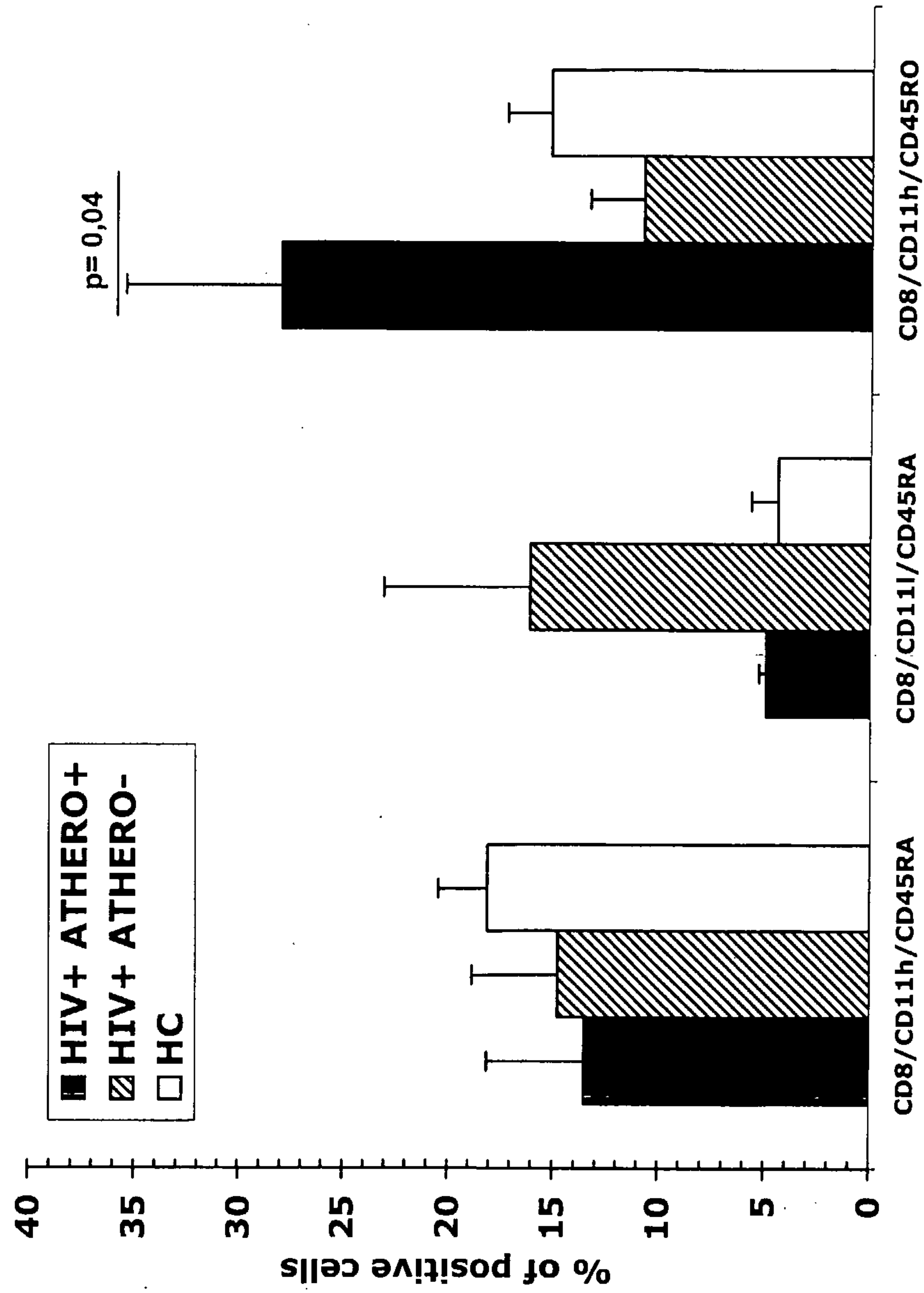
mean values $\pm$ SE

Fig. 1c

Fig. 1d



mean values $\pm$ SE



mean values + SE

Fig. 1e

6/14

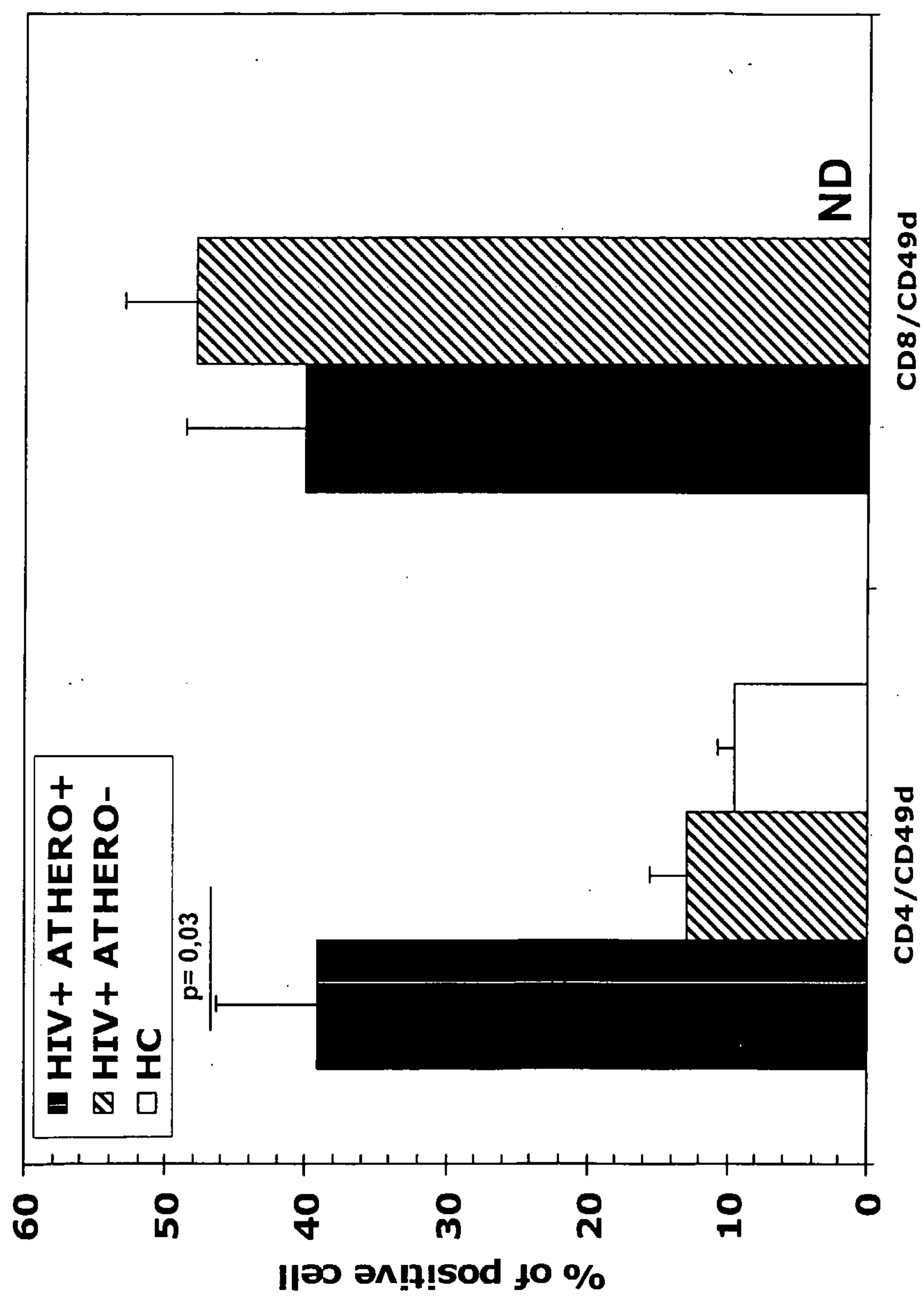
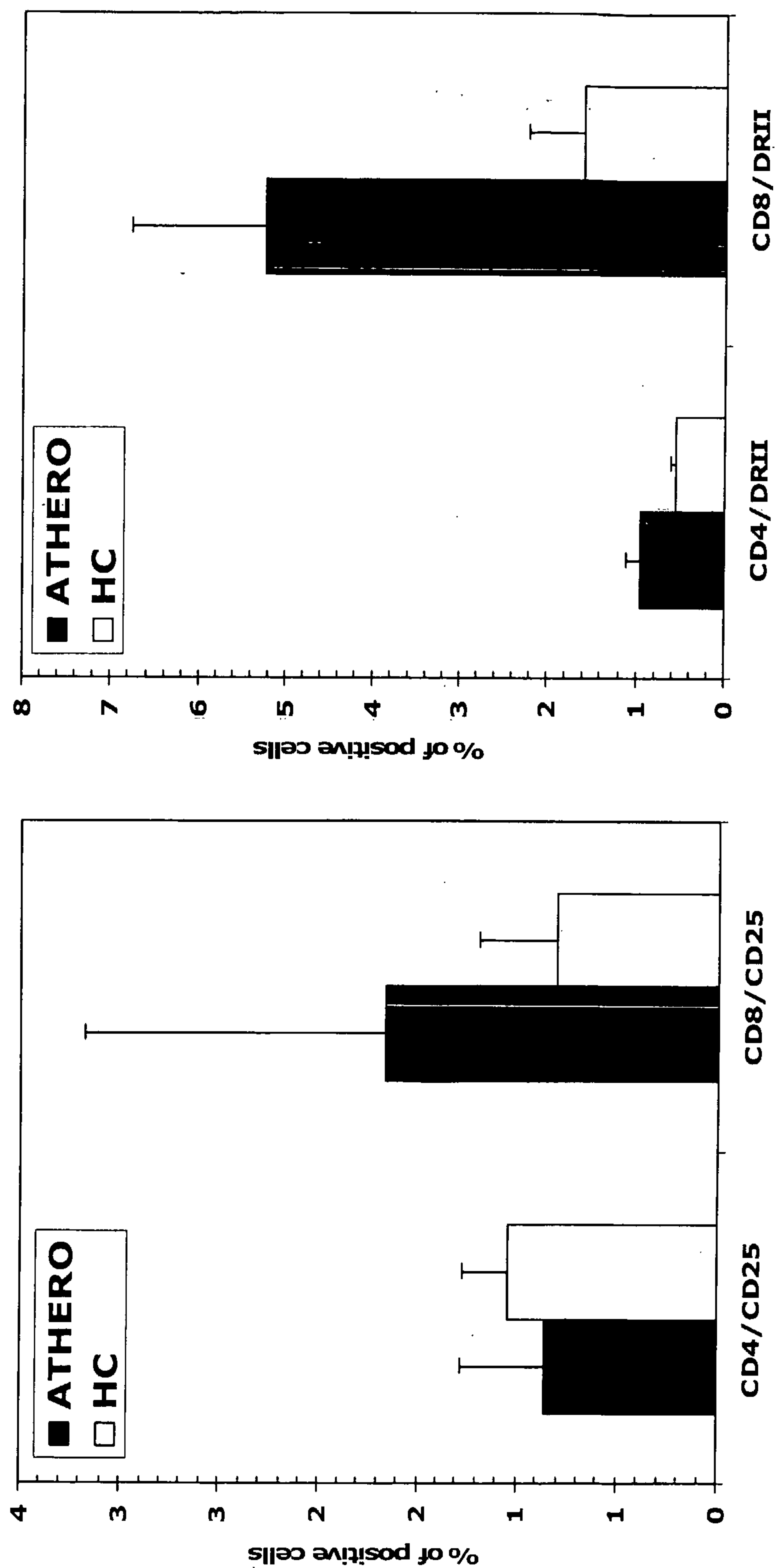
mean values $\pm$ SE

Fig. 1f

7/14

Fig 2.a



mean values $\pm$ SE

8/14

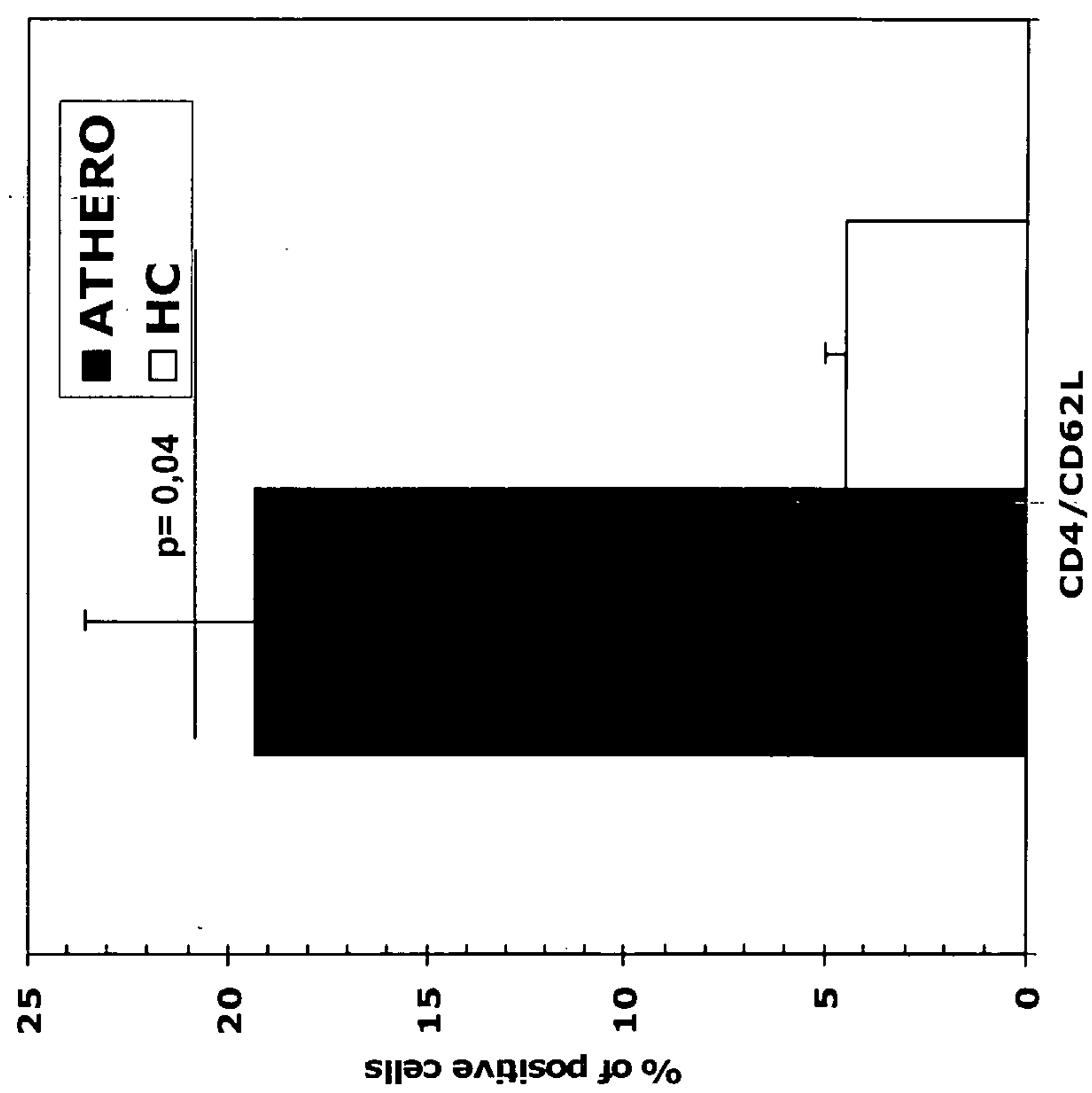
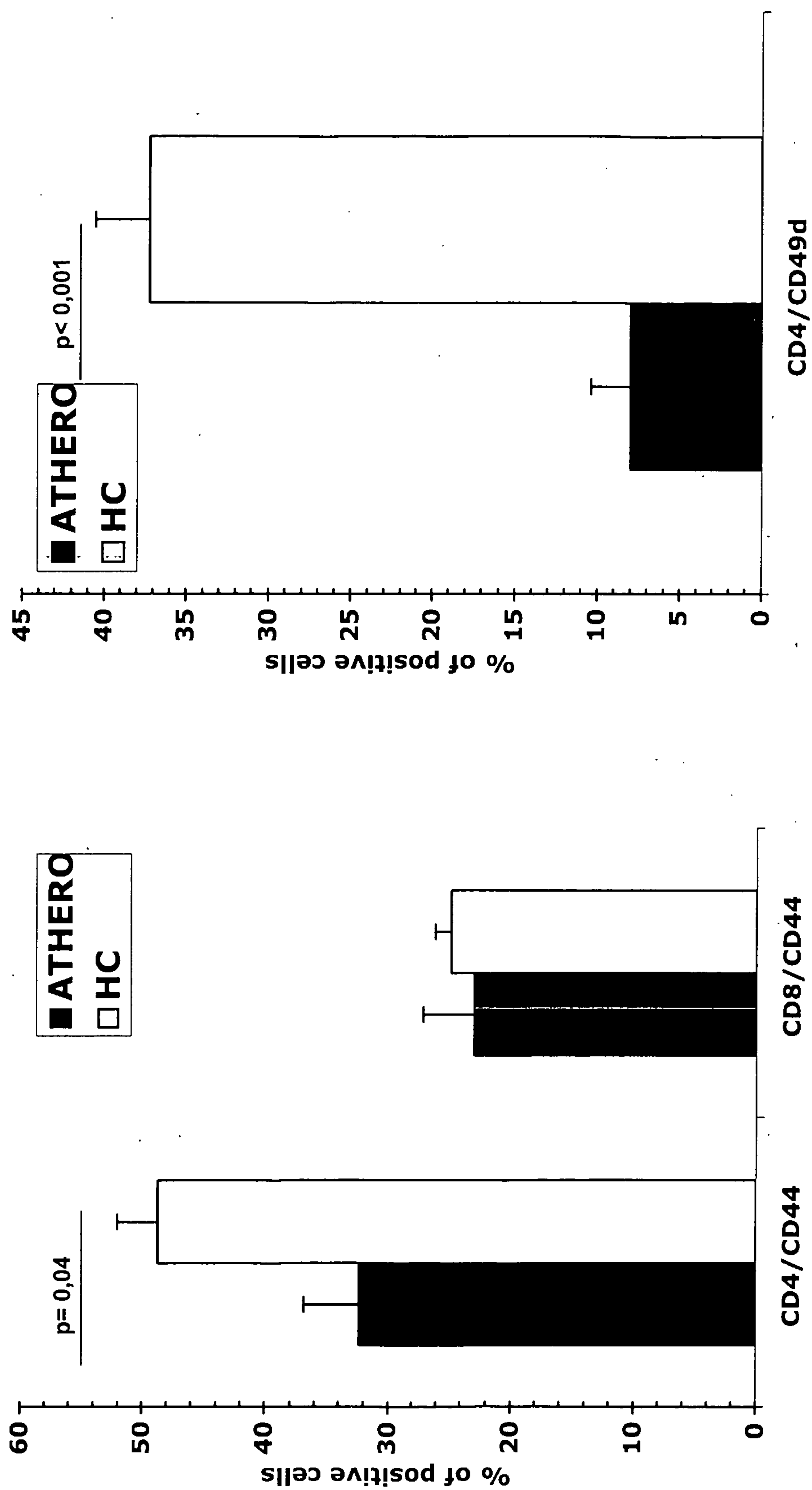


Fig 2. b

mean values $\pm$ SE

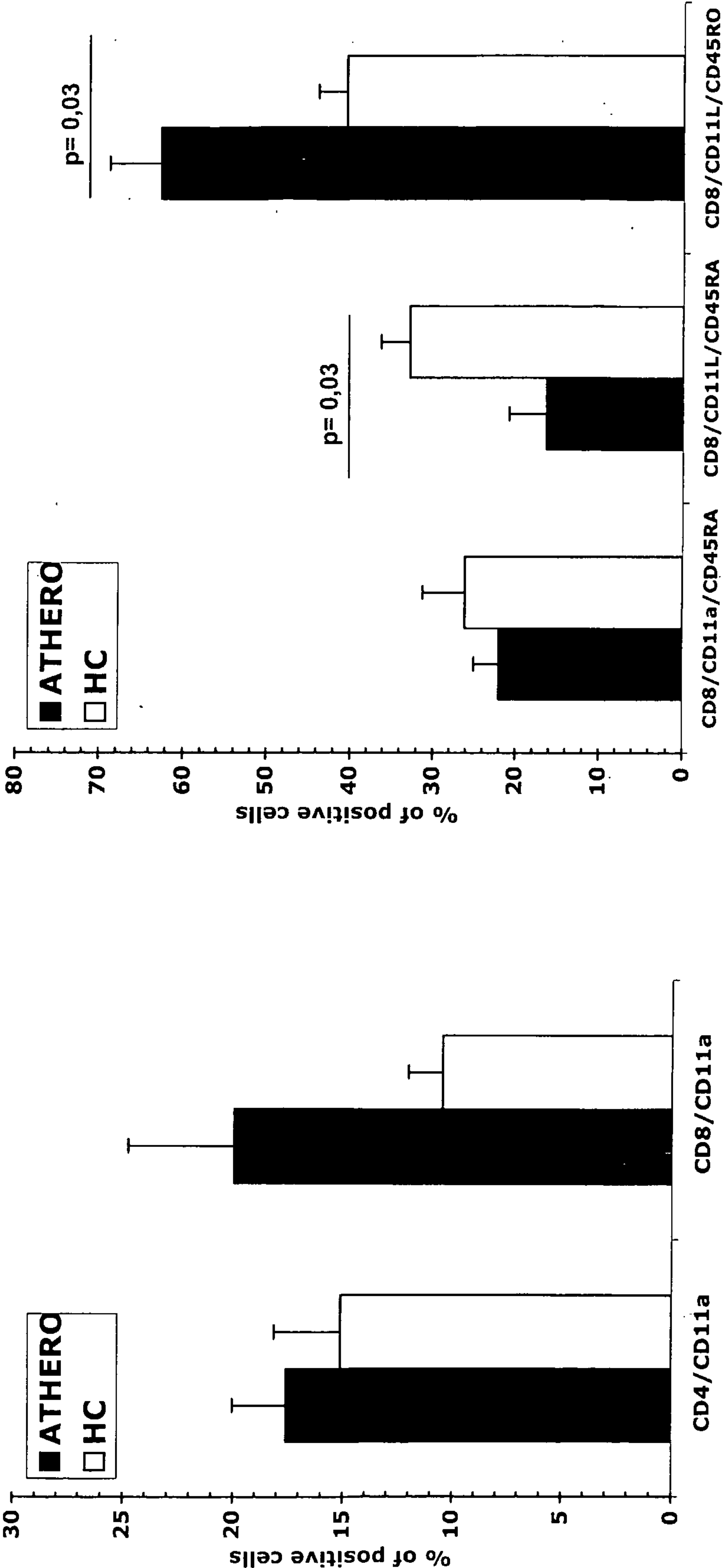
9/14

Fig 2. c

mean values $\pm$ SE

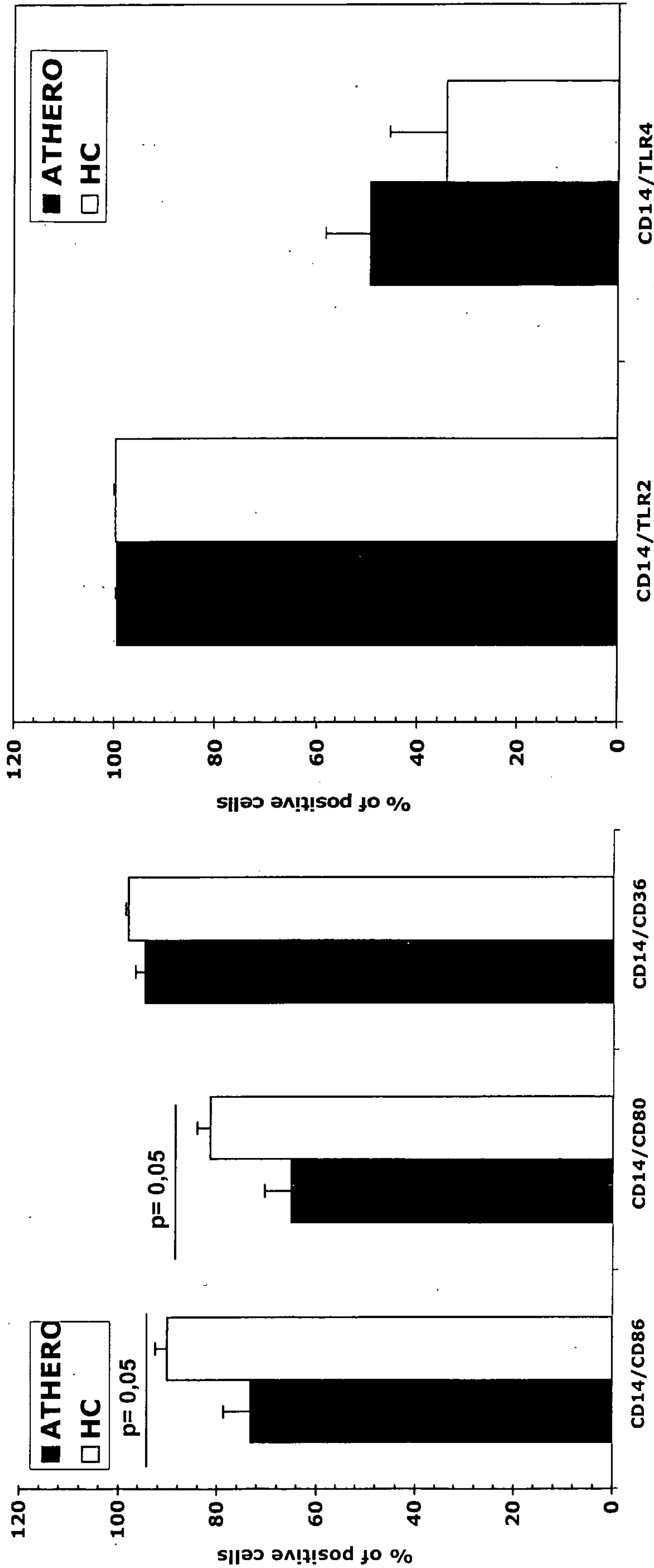
10/14

Fig 2. d



mean values ± SE

11/14

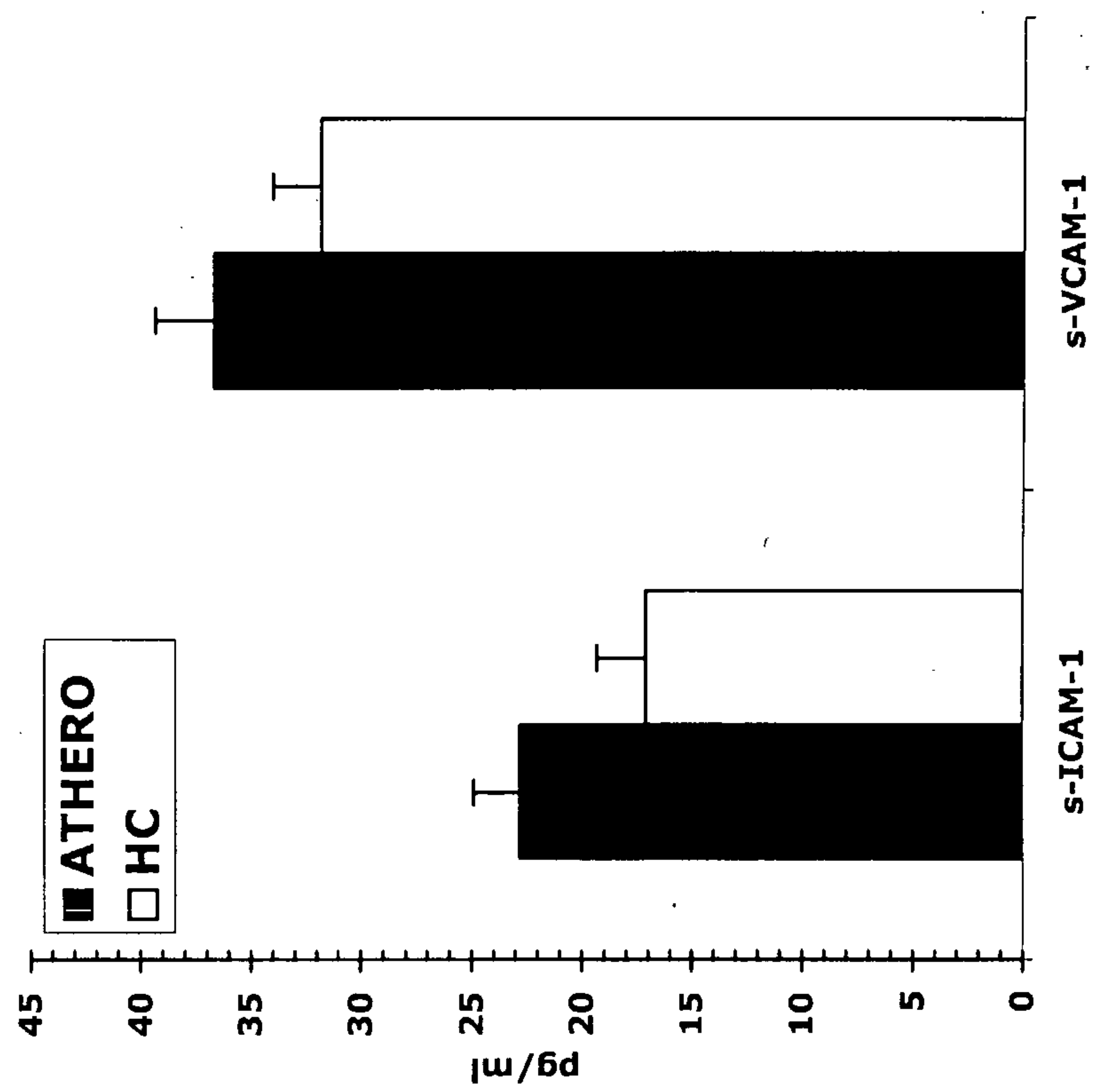


mean values $\pm$ SE

Fig 2. e

12/14

Fig 2. f

mean values $\pm$ SE

13/14

Fig 2. g

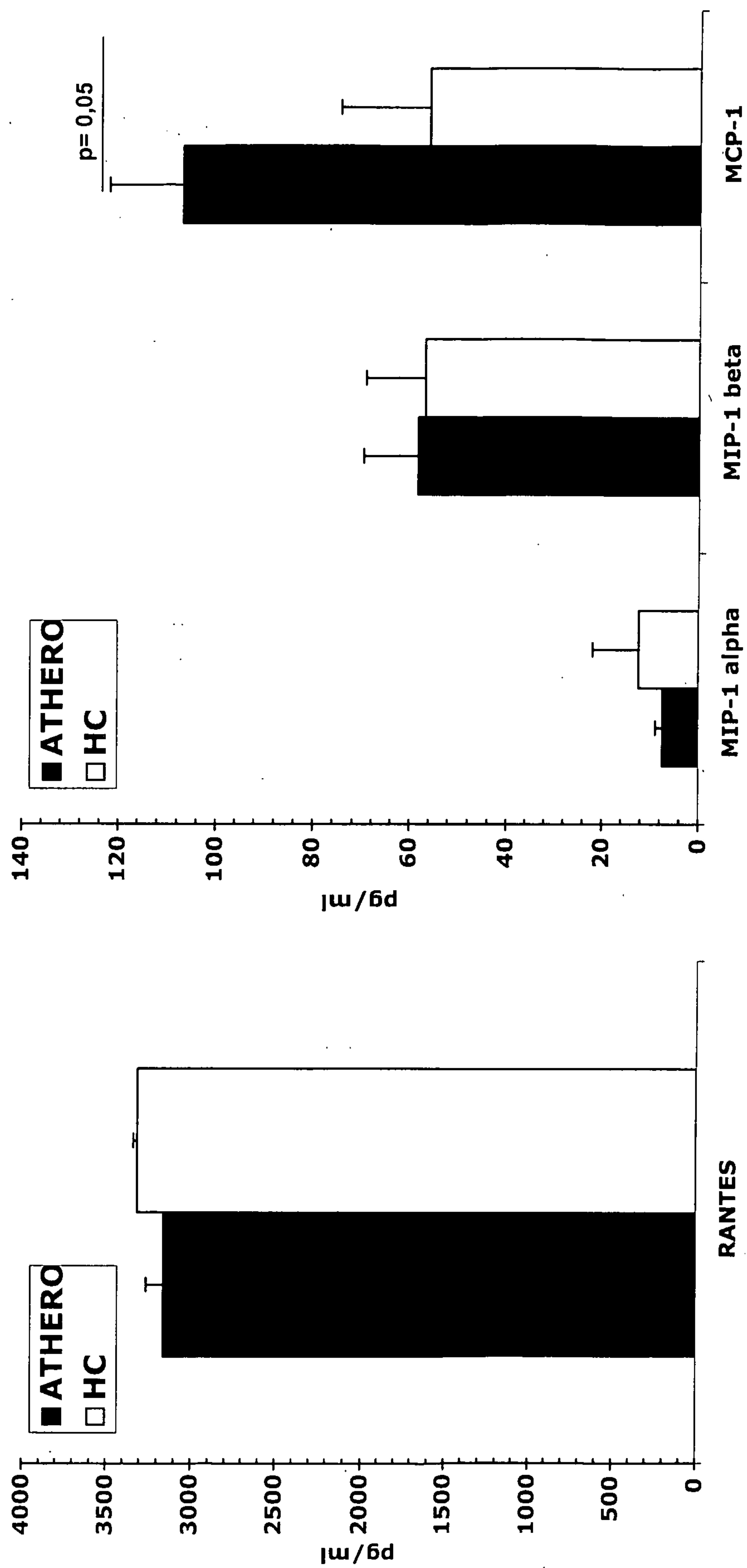
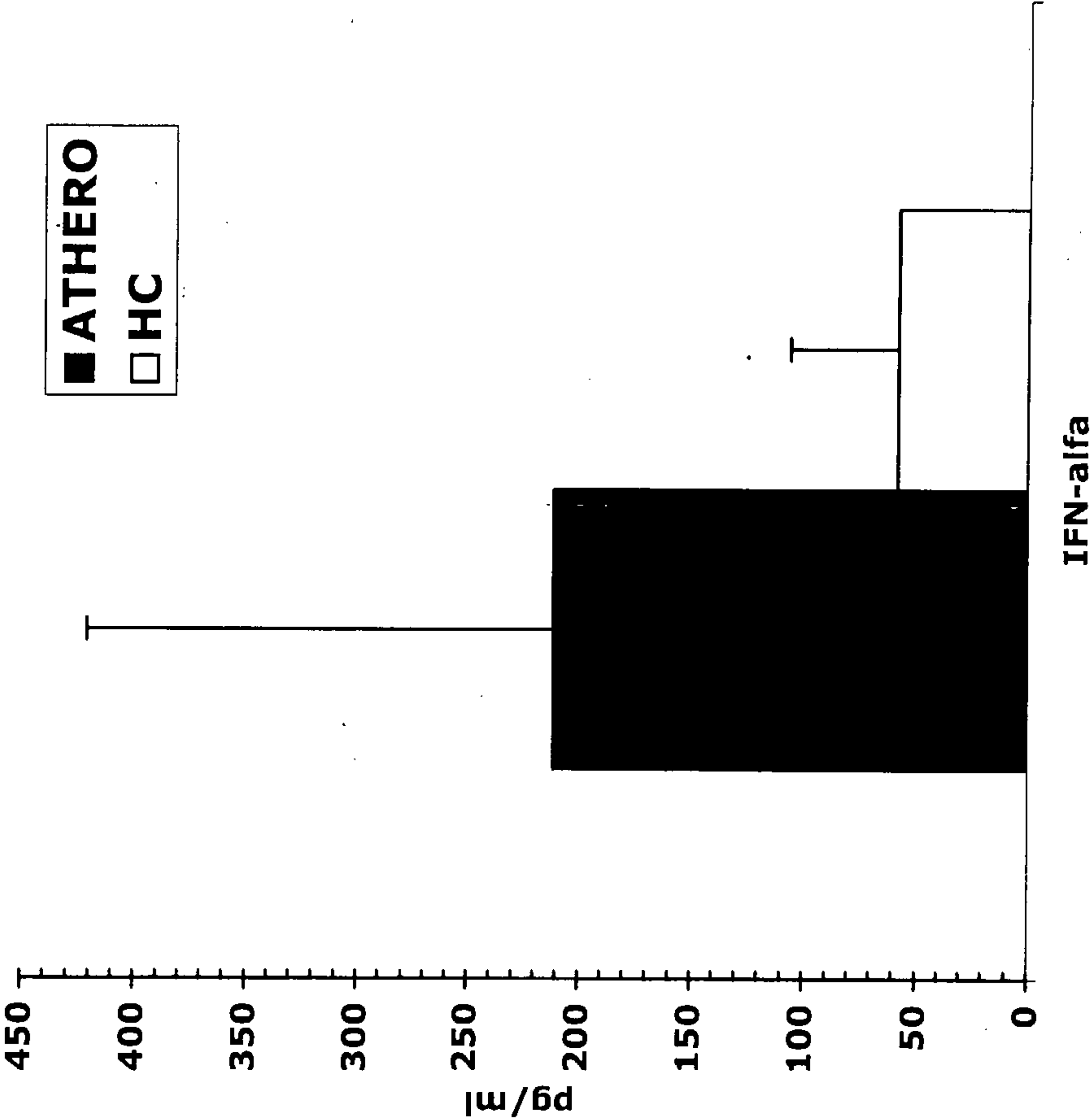
mean values $\pm$ SE

Fig 2. h



mean values $\pm$ SE