Peer reviewed article

Systemic Th1- and Th2-gene signals in atopy and asthma

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Summary

Background: Atopic disorders have been associated with a Th2-cytokine predominance. This study investigated Th1- and Th-2-related gene expression in asthmatics, atopics and healthy individuals.

Methods: We compared Th1- and Th2-related *in vivo*-signals using gene expression arrays in 18 atopic asthmatics, 8 atopic non-asthmatic and 14 healthy control subjects. Purified mRNA from peripheral blood mononuclear cells was reverse-transcribed and hybridised to cDNA membranes. Group differences were assessed after standardisation with the Mann-Whitney U-test.

Results: Atopic individuals had upregulated lymphotoxin-alpha and downregulated IFNGR1. On the other hand, they had particularly high IL4, IL5 and IL4R levels, together with significantly upregulated IL10. Asthmatic individuals had normal Th1-gene expression, but an upregulation of Th2-genes. Atopic individuals had high, asthmatic individuals excessively high IL12RB1-levels. No Th2-gene was downregulated in both atopic phenotypes. The expression of IL6R correlated with the daily dose of inhaled corticosteroids.

Conclusions: Atopic individuals had a down regulation of key Th1-genes and an upregulation of Th1- and Th2-genes, resulting in a balanced upregulation of Th-specific genes. In contrast, asthmatic subjects had normal Th1-gene expression but a constant upregulation of Th2-specific genes, leading to Th2-predominance.

Key words: asthma; atopy; gene expression; Th1; Th2; cytokines

Introduction

In 1986, Mosmann *et al.* [1] described the existence of two distinct murine CD4+ T cell populations. The two subsets of CD4+/helper T cell clones were divided according to the cytokine patterns that they produced. The first subset, T helper cell type 1 (Th1), produces IL-2 and interferon-γ and was found to mediate delayed-type hypersensitivity responses [2]. T helper cells type 2 (Th2) were found to produce IL-4 and IL-5 and to potentate antibody responses, influencing immunoglobulin isotype switching to IgE/IgG1 [3]. Since these early reports, the Th1/Th2 dichotomy has been documented *in vitro* and *in vivo* in a large number of studies in mice [4–8].

Most of the insight into Th-subsets has been derived from the murine model. In humans, the situation is less clear-cut. Most of the work in humans was done in T cells from healthy donors, which did not clearly fit into the Th1 or Th2 subsets. Romagnani et al. showed in an *in vitro* study that CD4+ T cell clones derived from patients with autoimmune diseases or conjunctivitis developed into the two distinct T cell clones with the same cytokine profile as in mice [9]. Recently, a large body of evidence has implied that atopic diseases are orchestrated by Th2 cytokine predominance [10]. Finotto et al. demonstrated that mice lacking the Th1-transcription factor T-bet spontaneously developed airway inflammation reminiscent of human asthma [11].

We have previously shown that cDNA microarrays are useful tools in detecting disease pathway signals in asthma and atopy [12–14]. However, no study looking at broad Th1- and Th2-related signals has been performed to date. The aim of this study was therefore to compare Th1- and Th2-related *in vivo* gene signals in atopy and asthma using gene expression arrays in peripheral blood mononuclear cells.

Funding: I.C.B. and L.J. were supported by the Uarda Frutiger Foundation, the Swiss National Foundation and the Novartis Jubilee Foundation of Switzerland.

Material and methods

Patients

Control subjects (C), atopic non-asthmatic (AN) and atopic asthmatic (AA) individuals were recruited from healthy hospital staff and the outpatient asthma clinic, respectively. C were defined as healthy individuals with a negative skin-prick test. Atopy was defined as a positive skin prick test (wheal of ≥3 mm diameter greater than the negative control wheal) to a range of 4 common aeroallergens: *Dermatophagoides pteronissinus*, cat, dog and mixed grasses (ALK, Denmark). Asthma was defined as documented bronchial hyperreactivity (methacholine challenge) or documented bronchial reversibility after inhalation of salbutamol in a sensitised and symptomatic individual. The asthmatic subjects were allowed to use inhaled corticosteroids. Smokers and those needing oral steroids in the previous 6 months were excluded. The severity of asthma was determined using Aas asthma severity score [15]. The Aas score is a five-step clinical score assessing events during the previous year. Asthmatic individuals were divided into mild/moderate asthmatics (Aas score ≤3) and severe asthmatics (Aas score >3). Subjects had to give informed consent. The study was approved by the local ethical committee.

PBMC preparation and cDNA hybridisation

Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation and washed in AIM-V serum-free culture medium [12]. The proportion of CD3-positive cells, thus lymphocytes, within these PBMCs was 80% or higher in a representative sample of FACS analyses. The purified mRNA populations (TRIzol™, Life Technologies Ltd, Palsley, UK and Oligotex™, Qiagen Ltd, Crawley, UK) were reverse transcribed with oligo-dT primer mix and labelled with ^{32}P-dATP (Amersham Life Science Ltd, Buckinghamshire, UK). The quantification was done via autoradiography and phosphor imaging following an overnight hybridisation onto membranes with immobilised probe cDNA for 609 gene products in duplicate (Atlas™, Clontech, Palo Alto CA, USA; www.clontech.com),

Statistical analysis

Data were analysed using SPSS for Windows 9.0.0 (SPSS Inc., Chicago IL, USA) statistical package. The signal intensity of the different gene products was standardised according to the GM100 method [12, 13]. Multiple Mann-Whitney U-tests were used to screen for differential gene expression in different phenotypes. Multiple Spearman's correlations were done to identify correlations between the expression of Th-specific gene products with the total daily dose of inhaled corticosteroids. A significance level of 5% was taken, however, a prudent interpretation was applied due to multiple significance testing in this exploratory approach. Wherever possible, dysregulated genes were validated by integration of gene expression results of up- or downstream genes of the respective signalling pathway.

Results

Table 1 displays subjects' characteristics. The subgroups were comparable in terms of age distribution. The results of the comparison of the expression of Th1- vs. Th2-specific genes are given in table 2 and figure 1. It can be concluded that AN and AA had upregulated expression of Th2-genes. For Th1-genes, AN showed a down regulation of IFNGR1 and an upregulation of LTA. AN had high, AA excessively high IL12RB1-levels. No Th2 gene was downregulated in AN and AA. AN had particularly high IL4, IL5 and IL4R levels. AA showed an upregulation of the same genes, however in a slightly attenuated form compared to AN. The Th2-inducing transcription factors STAT6 and GATA3 were not significantly different between the groups. The regulatory cytokine IL10 was significantly increased in AN but did not reach significance in AA. Genes which are known to be expressed by either Th1 or Th2 lymphocyte clones, especially IL6 and IL6ST, were found in higher levels in AN and AA. Th3-cytokines (TGFB1, TGFB2 and TGFB3) were expressed below the limit of detection.

When Th1- was compared to Th2-gene ex-

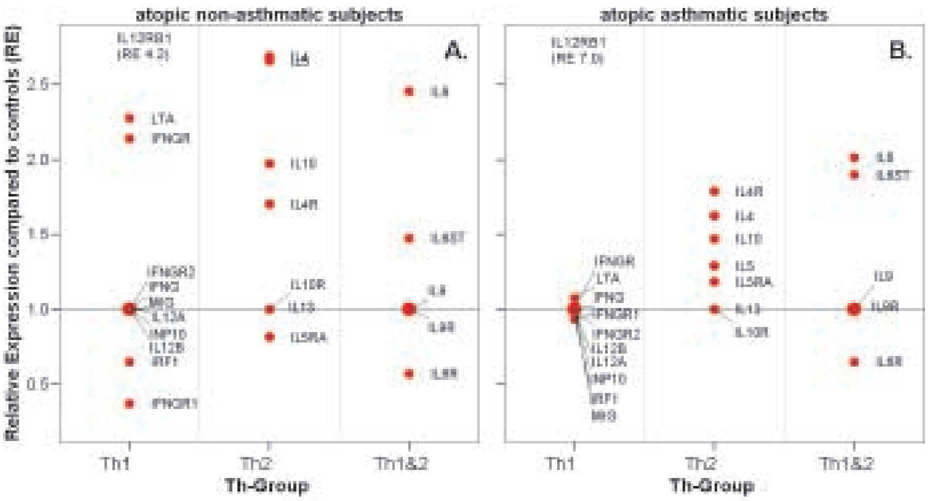


Figure 1

Scatter plot of the expression of Th1/2-cytokines and their receptors as found in atopic (AN) and asthmatic (AA) individuals. Expression levels of the genes are given relative to the expression in controls. Atopic individuals had a significant down regulation of IFNGR1 and a trend for a downregulated IRF1. LTA was upregulated. Asthmatics have normal Th1-gene expression apart from an IL-12 receptor component (IL12RB1). Both atopic and asthmatic individuals had clearly upregulated Th2-gene expression. Cytokine genes, which are expressed in Th1- and Th2-lymphocyte clones, were upregulated in atopic and asthmatic individuals. Th3-cytokines were expressed below the limit of detection and not shown on this graph. For significance levels consult table 2. Genes are labelled with their HUGO short names.

Table 1

Subject characteristics of healthy individuals and atopic subjects with and without asthma. ICS: inhaled corticosteroids, SPT: skin prick test, Aas score: Aas' clinical asthma severity score [22].
* Median [range]. NA: not applicable.

Demographics	Total (n = 40)	Healthy Controls (C) (n = 14)	Atopy (AN) (n = 8)	Atopy & Asthma (AA) (n = 18)
Age (y)	39 ± 12 [18–66]	42 ± 13 [27–65]	36 ± 9 [23–49]	41 ± 14 [18–66]
Sex (M/F)	15/25	2/12	7/1	6/12
ICS	16 (20%)	0 (0%)	0 (0%)	16 (89%)
SPT positive	26 (65%)	0 (0%)	8 (100%)	18 (100%)
Aas score (I/II/III/IV/V)	NA	NA	NA	2/1/4/9/2

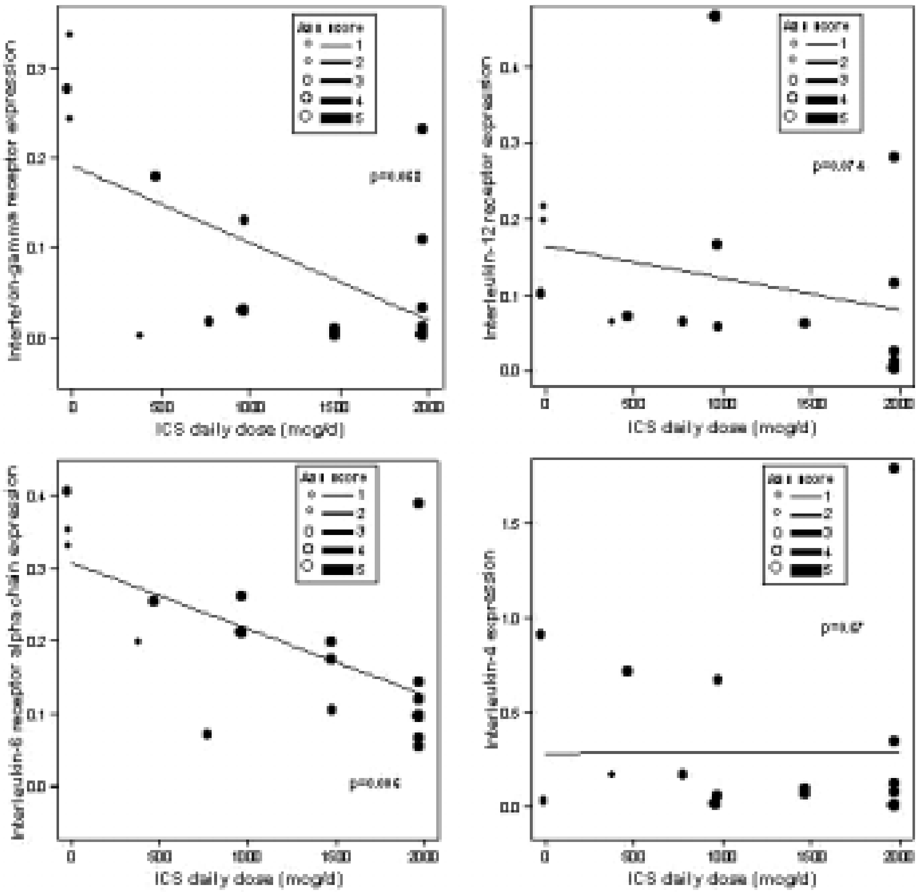


Figure 2

Scatter plots showing expression levels of Th-specific genes as a function of the inhaled corticosteroid dose in asthmatic individuals. The graph shows trends for Th1-specific genes (IFNGR, top left; IL12RB1, top right) and a clear correlation for IL6R alpha chain, a gene involved in IL6-signalling (Th1/2-gene, bottom left), to be downregulated by ICS. In contrast, there was no measurable effect in the Th2-genes tested (IL4 expression shown as an example, bottom right). The ICS dose is given as beclomethasone-equivalent dose. The gene expression levels show GM100-normalised expression levels (see methods).

pression, AN had a down regulation of one key Th1-gene and an upregulation of other Th1- and Th2-genes. AA had normal Th1-gene expression apart from a component of the IL12 receptor (IL12RB1), which was excessively high. On the other hand, in the AA group there was a very constant upregulation of Th2-specific genes and genes which can be expressed by both Th1- and Th2-cell clones in the AA group.

Inhaled steroids showed a significant negative correlation with the IL6R-expression (figure 2, table 2) and relevant trends for the receptors of the Th1-cytokines IFN-gamma and IL-12. There were no trends or correlations identified for Th2-gene expression and treatment or dose of inhaled corticosteroids.

Discussion

The Th1/Th2 paradigm has become an important issue in the pathogenesis of asthma and atopy [16]. In this study, we used cDNA array technology to analyse Th-profiles in individuals with asthma or atopy and in healthy controls. Atopic individuals with and without asthma had upregulated Th2-type cytokines compatible with an increased circulating Th2-lymphocyte number or increased Th2-type gene expression in atopy and asthma. These findings are in agreement with reports in the literature investigating expression of single cytokines on a protein level [17, 18].

In atopic asthmatic subjects Th1-cytokine expression was comparable to controls resulting in

Table 2

Gene expression results of Th1- and Th2-specific gene signals in unstimulated peripheral blood of healthy controls and atopic individuals with and without asthma. Non-parametric statistical tests were used (Mann-Whitney U-tests and Spearman-correlation). Acc. No: gene bank accession number, HUGO: Human Gene Nomenclature short names. Treg: regulatory T-lymphocyte genes.

Gene Acc. No	Name	HUGO	Expression relative to healthy			
			Atopy	p =	Asthma	p =
Th1-Genes						
X01992	Interferon-gamma	IFNG	Neg	Neg	Neg	Neg
A09781	Interferon-gamma receptor	IFNGR	2.14	0.151	1.01	0.675
J03143	Interferon-gamma receptor alpha chain	IFNGR1	0.37	0.003	0.94	0.382
X14454	Interferon regulatory factor [Interferon regulatory factor 1]	IRF1	0.65	0.088	0.94	0.621
M65291	Interleukin-12 alpha chain (Natural killer cell stimulatory factor, p35)	IL12A	Neg	Neg	Neg	Neg
M65290	Interleukin-12 beta chain (Natural killer cell stimulatory factor, p40)	IL12B	Neg	Neg	Neg	Neg
U03187	Interleukin-12 receptor	IL12RB1	4.08	0.001	6.95	0.001
D12614	Lymphotoxin-alpha [formerly tumor necrosis factor beta (TNF-beta)]	LTA	2.28	0.056	1.08	0.889
Th2-Genes						
M13982	Interleukin-4	IL4	2.69	0.114	1.63	0.223
X52425	Interleukin-4 receptor alpha chain	IL4R	1.71	0.056	1.79	0.030
X04688	Interleukin-5 [T-cell replacing factor]	IL5	2.66	0.17	1.29	0.79
M75914	Interleukin-5 receptor alpha chain	IL5RA	0.82	0.84	1.18	0.73
U16031	Transcription factor IL-4 STAT [Signal transducer and activator of transcription 6]	STAT6	1.08	0.55	1.26	0.21
X55122	Transcription factor GATA-3 trans-acting T-cell specific	GATA3	1.31	0.19	1.02	0.65
Treg-Genes						
M57627	Interleukin-10	IL10	1.98	0.040	1.47	0.239
U00672	Interleukin-10 receptor	IL10R	Neg	Neg	Neg	Neg
Th3(1&2)-Genes						
X04602	Interleukin-6 [B-cell differentiation factor]	IL6	2.46	0.005	2.02	0.009
M20566	Interleukin-6 receptor alpha chain	IL6R	0.57	0.785	0.65	0.494
M57230	Interleukin-6 receptor beta chain [membrane glycoprotein gp130]	IL6ST	1.48	0.113	1.90	0.085

clear overall Th2-predominance. In contrast to asthmatics, LTA, a Th1-cytokine, was also upregulated in atopic individuals without asthma. This might suggest a more balanced situation with Th1- and Th2-cell clones still competing for predominance in patients with atopy but not in asthmatics. Interestingly, IL10 was significantly upregulated in atopic subjects, but did not reach significance in asthmatic individuals. As a regulatory cytokine IL10 would clearly have the potential to lead to a loss in control of the inflammatory drive when reduced in concentration. On a gene expression level and for the genes tested it appears that there are only gradual differences between atopic individuals with and without asthma.

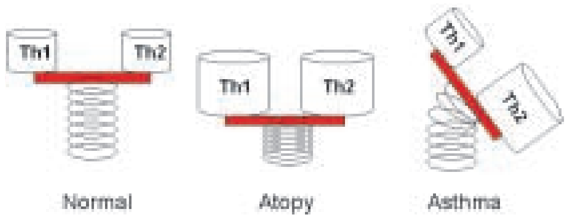
IFNGR1 was clearly downregulated in atopic individuals and there was a trend for an IRF1-downregulation. Interferon regulatory factor-1 (IRF1) functions as a transcriptional activator for the type I IFN genes [19] and has a role in the regulation of cell growth and differentiation. A recent study showed a significant linkage between the IRF1 micro-satellite marker and atopic phenotypes [20]. Furthermore, Nakao et al. found a significant association with an IRF1 polymorphism and childhood atopic asthma in a Japanese population [21]. The results of our study confirm the important role of IRF1 as a candidate gene for atopic phenotypes and support the IFNG-deficiency hypothesis for the development of atopy [22].

It has been postulated that the Th1 cytokine IFNG, acting through its heterodimeric receptors, IFNGR1 and IFNGR2, in the induction/proliferation of Th1 cells, might suppress the Th2 responses. Several dysfunctional mutations have been identified in interferon gamma receptor genes (IFNGR1 and IFNGR2) in relation to severe and selective infections with poorly pathogenic organisms [23, 24]. An intronic variant of IFNGR1 was associated with total serum IgE levels in a British population [25]. On the other hand, IFNGR1 and IFNGR2 gene polymorphisms showed no association with atopic asthma in Japanese children [21]. Our results confirm a possible role in the pathogenesis of atopy. However, further studies are needed to elucidate the involved mechanisms.

We found an upregulation of the relative expression of the Th1 cytokine receptor IL12RB1 in atopics and even more prominently in asthmatics. Interleukin-12 promotes cell-mediated immu-

Figure 3

Schematic diagram of Th cytokine expression. Atopic individuals show a harmonic upregulation of both Th1 and Th2-cytokines. Asthmatics have a relatively normal Th-1 cytokine expression and an elevated Th2-cytokine expression, resulting in a net Th2 predominance.



nity to intracellular pathogens by inducing Th1 responses and interferon-gamma production [26, 27]. IL12 binds to high-affinity beta-1/beta-2 heterodimeric IL12 receptor complexes on T cells and natural killer cells [28]. IL12RB1 deficiency has been associated with impairment of mycobacterial immunity [29] and severe mycobacterial and Salmonella infections [30]. It would be possible, but remains speculative in the absence of respective studies, that increased receptor levels correspond to a compensatory upregulation in case of a reduced IL12 signalling. We could not detect IL12 gene signals in either phenotype and, thus, cannot contribute to this hypothesis.

IL6 and its signal transducer IL6ST are produced by both Th1- and Th2-cells. We could demonstrate an upregulation of IL6 and IL6ST in both asthmatics and atopics and a compensatory down regulation of its receptor. This confirms previous reports in the literature. Modulation of IL6 levels by Th2-type cytokines has been shown to play a role in allergic reactions through the IL-6 promoting effect on IL4 mediated IgE production [31]. Levels of IL6 are elevated in asthmatics during asthma attacks [32] or following antigen challenge [33] suggesting an importance in the pathophysiology of asthma.

At the time the study was designed, inhaled corticosteroids were thought to have no measurable systemic effects on circulating lymphocytes. For this and also for ethical reasons, patients with moderate to severe asthma were not asked to discontinue their treatment. This is the first recorded observation of progressively reduced IL6R-levels with increasing daily doses of inhaled corticosteroids and the same trends for IFNGR and IL12RB1 (Fig. 2). Due to the design of the study, however, we cannot exclude a bias caused by asthma severity. Interestingly, no Th2-specific gene correlated with inhaled corticosteroids. Thus, our analysis provides evidence that Th1-cytokine signalling might be preferentially downregulated by steroids whilst Th2-gene signals remain unchanged. This observation is in agreement with the observation that steroids generally do not lead to a persistent remission of asthma [34]. A second point underlining the validity of this observation is the fact that there are only contradictory results concerning the effect of steroids on total IgE-levels [35–37]. However, our study sample is too small to analyse the effects of inhaled corticosteroids on gene expression and, thus, has to be regarded as preliminary.

Due to the nature of our approach, i.e. measuring gene expression in peripheral blood, it is not possible to estimate the importance of local factors in the lungs of asthmatic individuals, which is a limitation of the current study. Nevertheless, the blood circulates approximately once per minute through the lungs and sufficient "spill-over" of local phenomena might occur and has been described [38]. The main goal of the study was to analyse the *in vivo*-situation, where dysregulated genes are likely to contribute to the phenotypic presentation. Many of the differences in gene expression identified in our *in vivo*-study were rather small in terms of up- or down regulation. It is difficult to assess the biological significance of differences in gene expression. Due to a limited reproducibility of classical RT-PCR with gel electrophoresis, the expression should at minimum be doubled in order to validate the results as a positive finding. With new technology, including gene arrays and real-time PCR, smaller differences can be picked up reliably. Furthermore, from a statistical point of view it is better to compare the expression variability of phenotypes A with B, thus performing statistical significance testing. From a biological point of view, it seems reasonable to postulate that the expression of highly potent cytokines, like IL4 and IFNG, is regulated in a very tight band in order to maintain a physiologic homeostasis of pro- and anti-inflammatory signals. Thus, in our view, an upregulation in gene expression of 50% in an *in vivo*-system might potentially have great biological significance.

In conclusion, this *in vivo*-study provides evidence that key Th1 genes are downregulated in atopic individuals and that Th2-cytokines are upregulated in asthma and atopy (Figure 3). With this analysis, it was possible to weigh the expression of different cytokines against each other and, thus, to estimate their potential importance *in vivo*. The role of IRF1, IFNGR1 and IL12RB1, potential candidate genes for atopy and asthma, needs to be further evaluated.

We thank Prof. Tony Hegarty and Dr. Jacky Ohanian for the permission to use their phosphor imager. Many thanks as well to Frazer Smillie for technical advice. I.C.B. and L.J. were supported by the Uarda Frutiger Foundation, the Swiss National Foundation and the Novartis Jubilee Foundation of Switzerland.

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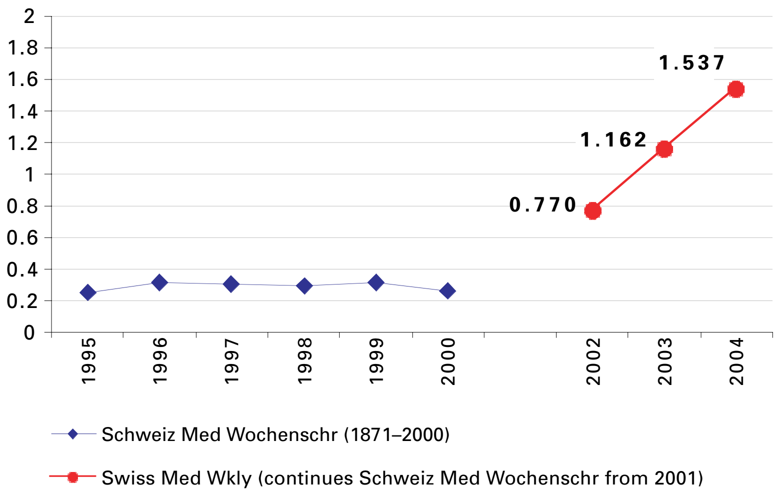

EMH
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All manuscripts should be sent in electronic form, to:

EMH Swiss Medical Publishers Ltd.
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