Detecting oxysterols in the human circulation

To the Editor:

Oxysterols are oxygenated metabolites of cholesterol. On the basis of *in vitro* experiments, oxysterols have been ascribed many regulatory roles in connection with inflammation, neurodegeneration and atherosclerosis[1]. They are present in trace amounts in biological systems, and their analysis represents a challenging analytical problem.

The 15-oxygenated cholesterol species 5α-cholest-8(14)ene-3β,15α-diol and 3β-hydroxy-5α-cholest-8(14)-en-15-one have been chemically synthesized from 7-dehydrocholesterol. Although commercially available, these have not been identified before in humans, and an enzymatic pathway for their synthesis has not been defined. Farez *et al.* have reported in *Nature Immunology* that these two sterols occur in the human circulation at concentrations considerably higher than those of any other endogenous oxysterol[2]. They report higher concentrations in patients with multiple sclerosis in the progressive phase. On the basis of their findings, the authors did extensive *in vitro* experiments in which they exposed cultured cells to the same high concentrations of 5α-cholest-8(14)ene-3β,15α-diol as they found *in vivo* (1,000 ng/ml). They concluded that 15-oxygenated sterols may be important factors in pathogenic mechanisms that underlie multiple sclerosis.

We represent three independent research groups with decades of experience in the analysis of oxysterols. We have been unable to confirm the findings reported by Farez *et al.* in terms of the presence of 15-oxygenated sterols in human plasma or serum.

One of our groups (I.B. and U.D.) has used combined gas chromatography–mass spectrometry to analyze plasma from 40 healthy Swedish volunteers without finding substantial amounts of the two 15-oxygenated sterols noted above[3]. In collaboration with a clinical group (T.O.), they analyzed plasma from 33 Swedish patients with multiple sclerosis at various stages of disease, again without detecting substantial concentrations of the sterols noted above. Successful recovery experiments excluded the possibility of loss of 15-oxygenated sterols during extraction and workup procedures[3].

The second group (D.W.R. and J.G.M.) has used combined liquid chromatography–mass spectrometry (a modification of a published method[4]) to analyze more than 1,200 subjects in Dallas (USA) who represent a broad cross-section of ethnicities, social strata and health status. Using these methods, these researchers found that none of the subjects had substantial concentrations of 15-oxygenated sterols in the circulation (D.W.R. and J.G.M., data not shown).

The third group (W.J.G. and Y.W.) has used derivatization combined with liquid chromatography–high-resolution mass spectrometry and multistage fragmentation to analyze more than 40 plasma samples from healthy and sick infants and adults[5]. They did not find substantial concentrations of 15-oxygenated steroids in any of the plasma samples (W.J.G. and Y.W., data not shown).

We suggest that 15-oxygenated steroids should not be used as biomarkers in connection with multiple sclerosis and that a pathogenic role for these sterols must be reconsidered.

Ingemar Björkhem[1], Ulf Diczfalusy[1], Tomas Olsson[2], David W Russell[3], Jeffery G McDonald[3], Yuqin Wang[4] & William J Griffiths[4]

[1]Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Huddinge, Sweden. [2]Department of Clinical Neuroscience, Karolinska Institutet, Karolinska University Hospital Solna, Sweden. [3]Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas, USA. [4]Institute of Mass Spectrometry, School of Medicine, Swansea University, Swansea, UK.
e-mail: ingemar.bjorkhem@karolinska.se

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Björkhem, I. & Diczfalusy, U. *Arterioscler. Thromb. Vasc. Biol.* **22**, 734–742 (2002).
2. Farez, M.F. *et al. Nat. Immunol.* **10**, 958–964 (2009).
3. Björkhem, I. *et al. J. Lipid Res.* **52**, 170–174 (2011).
4. McDonald, J.G., Thompson, B.T., McCrum, E.C. & Russell, D.W. *Methods Enzymol.* **432**, 145–170 (2007).
5. Griffiths, W.J. *et al. J. Proteome Res.* **7**, 3602–3612 (2008).

Farez *et al.* respond:

We thank Björkhem and colleagues for their comments on our report of oxysterols in the blood of patients with multiple sclerosis[1] and for applying their considerable expertise to this question. We address below the issues they raise about their inability to demonstrate the presence of substantial concentrations of 15-oxygenated C27 steroids in human circulation.

The analysis of oxysterols in biological samples is challenging because of the presence of cholesterol and other, more abundant lipids[2]. Consequently, the specific method used to detect oxysterols can have a considerable effect on the oxysterol concentrations measured in blood[3,4]. For our studies, we used a method similar to a published procedure[5] to analyze the concentration of the oxysterol 7-ketocholesterol in cerebrospinal fluid and serum samples from patients with multiple sclerosis and mice with experimental autoimmune encephalomyelitis (EAE; **Supplementary Methods**). This method included several preparatory steps before the analysis of serum samples by gas chromatography–mass spectrometry, including lipid extraction, the addition of an internal standard (5α-cholestane) and fractionation with Diol columns. We then analyzed the samples on a gas chromatograph coupled with a mass spectrometer. We based peak identification on retention time and ion mass spectrometry relative to those of external standards and quantified these by integration relative to the internal standard (**Supplementary Methods**). We did several validation controls to assess the performance of this procedure. For example, 15α-hydroxycholesterol and 7β-hydroxycholesterol could