EXTENDED REPORT

Loss of phosphatase and tensin homolog (PTEN) in myeloid cells controls inflammatory bone destruction by regulating the osteoclastogenic potential of myeloid cells

Stephan Blüml,1 Martin Friedrich,2 Tobias Lohmeyer,2 Emine Sahin,2 Victoria Saferding,1 Julia Brunner,2 Antonia Puchner,1 Peter Mandl,1 Birgit Niederreiter,1 Josef S Smolen,1 Gernot Schabbauer,2 Kurt Redlich1

Handling editor Tore K Kvien

ABSTRACT

Objective Local bone destruction in rheumatic diseases, which often leads to disability and severely reduced quality of life, is almost exclusively mediated by osteoclasts. Therefore, it is important to understand pathways regulating the generation of osteoclasts. Here, we analysed the impact of the Phosphoinositide-3-Kinase (PI3K)/Phosphatase and tensin homolog (PTEN) axis on osteoclast generation and bone biology under basal and inflammatory conditions.

Methods We analysed osteoclastogenesis of wildtype (wt) and PTEN−/− cells in vitro and in vivo, pit resorption and qPCR of osteoclasts in vitro. Mice with a myeloid cell-specific deletion of PTEN and wt littermate mice were investigated by bone histomorphometry and clinical and histological assessment in the human tumour necrosis factor (TNF)-transgenic (hTNFtg) arthritis model.

Results We show that myeloid-specific PTEN−/− mice displayed increased osteoclastogenes in vitro and in vivo compared to wt mice. Loss of PTEN did not affect the generation or survival of osteoclast precursor cells. However, PTEN deficiency greatly enhanced receptor activator of nuclear factor κ-B ligand (RANKL)-induced expression of the master transcription factor of osteoclastogenesis, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), resulting in markedly increased terminal differentiation of osteoclasts in vitro. We also observed increased osteoclastogenesis under inflammatory conditions in the hTNFtg mouse model of arthritis, where hTNFtg/myeloid-specific PTEN−/− mice displayed enhanced local bone destruction as well as osteoclast formation in the inflamed joints. The extent of synovial inflammation, however, as well as recruitment of osteoclast precursor cells was not different between wt and myeloid-specific PTEN−/− mice.

Conclusions These data demonstrate that loss of PTEN and, therefore, sustained PI3-Kinase signalling in myeloid cells especially, elevates the osteoclastogenic potential of myeloid cells, leading to enhanced inflammatory local bone destruction. Therefore, although our study allows no direct translational conclusion since we used a conditional knockout approach, the therapeutic targeting of the PI3-Kinase pathway may be of benefit in preventing structural joint damage.

INTRODUCTION

Local and systemic bone loss is a hallmark of diseases such as osteoporosis, rheumatoid arthritis or spondylarthrit and often leads to disability and severely reduced quality of life and is therefore a serious health burden in humans.5–7 Such bone loss is almost exclusively mediated by a specialised type of cell, the osteoclast (OC).4–5 OCs are of haematopoietic origin and are derived from monocyte precursor cells.5 Many factors regulating differentiation and function of OCs have been described.6 7 Among them, the receptor activator of nuclear factor κ-B ligand (RANKL)-RANK pathway, as well as the macrophage colony-stimulating factor (M-CSF)-M-CSF-receptor system, have been found to be essential for the generation of OCs in vitro and in vivo conditions.8–17 A number of osteoclastogenic signalling pathways are integrated by the transcription factor nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), which regulates many OC-specific genes.12 13 Nonetheless, the generation and function of OCs is not fully understood and several factors that influence these processes are yet to be elaborated.

Phosphoinositide-3-kinases (PI3Ks) are heterodimeric complexes, comprising a regulatory subunit and a p110 catalytic subunit that phosphorylate the membrane phospholipid phosphatidylinositol (PI).14 The resulting activation of protein kinase B (Akt) as well as of glycogen synthase kinase 3 β (GSK3β) and the subsequent activation of genes regulated by these kinases mediate a variety of mechanisms such as inflammation, cell survival, cell migration, proliferation and cytoskeletal remodelling.14–16

Phosphatase and tensin homolog (PTEN) is a 53 kD phospholipid phosphatase, whose main substrate is PI(3,4,5)P3, the principal second messenger of the PI3K pathway. Therefore, PTEN is generally regarded as an antagonist of PI3Ks. PTEN is a potent tumour suppressor,19–21 and recent findings support the notion that the PI3K/PTEN pathway is intricately involved in the modulation of innate immune responses.19–21 Additionally, PTEN seems to be responsible for chemokine-dependent directed migration of various cell types, and also plays a major role in angiogenesis.22 23 PI3K has been shown to play a role in osteoclastogenesis, as demonstrated by the fact that SH2 domain-containing inositol S'-phosphatase (SHIP) deficient mice are osteoporotic due to increased generation of OCs.24 25 Additionally, inhibition of PI3K
reduces osteoclastogenesis in vitro. PTEN has also been implicated in osteoclastogenesis, since transfection of RAW264.7 macrophages with a dominant negative version of PTEN increased, whereas overexpression of functional PTEN reduced osteoclastogenesis.

Selective lack of PTEN in osteoblasts leads to enhanced bone formation as a result of prolonged survival and increased function of osteoblasts. However, the effect of PTEN deficiency on OC precursors or OCs in vivo, has not been investigated yet. We therefore analysed the effect of PTEN deficiency on osteoclastogenesis under homeostatic and inflammatory conditions by employing a conditional knockout mouse that lacks PTEN expression selectively in myeloid cells, subsequently referred to as myeloid-specific PTEN fl/fl mice.

MATERIALS AND METHODS

Animals

Animals were identified by PCR from tail DNA using the following primers: human tumour necrosis factor (hTNF) transgene construct: 5′-TACCCCTCCCTTGACAGGACC-3′ and 5′-GCCCTTCA TAATATCCCCCA-3′; Clinical signs of arthritis and body weight were determined once weekly. Animals were killed by cervical dislocation 12 weeks after birth. All animal procedures were approved by the local ethical committee. PTEN fl/fl mice were provided by Dr Tak W Mak (University Health Network, Toronto, Canada). To selectively reduce PTEN expression in myeloid cells, PTEN fl/fl mice were crossed with mice expressing the Cre recombinase under the control of the Lysozyme M (LysM) promoter (provided by Dr R Johnson, University of California San Diego, La Jolla, California, USA) to generate LysMcrePTEN fl/fl mice (myeloid pten fl/fl) mice. LysMcrePTEN fl/fl mice and PTEN fl/fl mice were backcrossed at least eight generations onto the C57BL/6J background. These mice were crossed into Tg197 hTNF transgenic mice (hTNFtg; genetic background C57BL6-129) to obtain LysMcrePTEN fl/fl mice. Animals were identified by PCR from tail DNA using the following primers:

Clinical assessment of arthritis

Clinical signs of arthritis, including grip strength and paw swelling were assessed weekly in mice starting 4 weeks after birth. Paw swelling was assessed by using a well-established semiquantitative score: 0=no swelling, 1=mild swelling of the toes and ankle, 2=moderate swelling of the toes and ankle, and 3=severe swelling of the toes and ankle. Grip strength of each paw was analysed on a wire mesh (3-mm in diameter) using a well-established semiquantitative score from 0 to 3=severely reduced grip strength).

Histological sections and histochemistry

Hind paws were fixed and stained as previously described. Quantification of the areas of inflammation, H&E sections were evaluated using an Axioskop 2 microscope (Carl Zeiss MicroImaging) and Osteomeasure Analysis System (Osteometrics). Tissue sections were stained with rat monoclonal antimacrophage (F4/80) antibody (Ab) (Serotec, Oxford, UK); diluted 1:300, followed by a biotinylated rabbit antirat IgG secondary Ab (Vector, Burlingame, California, USA). Proportions of F4/80+ OC precursors in hind paws as well as expression levels of NFATC1 in OCs were analysed using the TissueQuest software (Tissuegностics, Vienna).

Bone histomorphometry

Histomorphometry was performed on methacrylate-embedded un-decalcified plastic sections after von Kossa and Goldner staining. Quantifications were performed by digital image analysis (OsteoMeasure).

Dynamic labelling of bone

At 16 weeks of age, mice were given two injections of calcein green (Sigma–Aldrich) (30 mg/kg) 5 days apart. Left tibial bones were embedded in methoxymethylmethacrylate. Measurements were performed on the entire marrow region within the cortical shell using OsteoMeasure, and the mineral apposition rate (MAR) (μm/day) was calculated.

Statistical analysis

Data are given as mean±SEM. Group mean values were compared by using the unpaired two-tailed Student t test.

Ex vivo osteoclastogenesis

Bone marrow cells (BMC) were isolated and cultured for 3 days in 100 ng/mL M-CSF to enrich for monocytes/macrophages, and were then cultured in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium (FCS/DMEM) supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL (both from R&D Systems McKinley Place NE, Minneapolis) for another 3–4 days. OCs were defined and detected as tartrate-resistant acid phosphatase (TRAP)+ multicellular clusters (≥3 nuclei). Wörtmann was added to the culture at a concentration of 1 μM. Bone resorption was carried out on 0.4 mm-thick bovine cortical slices. The area of resorption per OC was calculated by dividing the total area of resorption by the total number of OCs using the Osteomeasure software.

qPCR

Total RNA was isolated from cultivated OCs using the RNeasy Mini kit (QiAGEN). 1 μg total RNA was used for first strand cDNA synthesis (Amersham Biosciences) and 1 μL cDNA was then used for PCR using the following primers: NFATc1: 5′-GACGACATCGGGAGAAGA-3′ and 5′-AGCCTTCCACGAAAATGA-3′; cathepsin K: 5′-GGAAGAACGTCCACGAGA-3′ and 5′-GTACTATAGCCGGCTCTCCACAG-3′; matrix metalloproteinase (MPM)-9: 5′-CCTGTGTTGTCCGTTACATC-3′ and 5′-CGCTGGAATGTCTAAGCGCGGGGA-3′; TRAP: 5′-ACAGCCC CACCTCCACCTC-3′ and 5′-TACGGGTCTCCTGGTCTCCCT TTGG-3′; calcitonin receptor: 5′-CATTTGGTCATTGTCCTGC-3′ and 5′-AGCCAATAGGAAGAAGGGCG-3′; and β-actin: 5′-TTGTT ATGTTGGAATGTTGCGAC-3′ and 5′-CTTGATGTCGCGCCGAC GTATTCC-3′. Quantitative RT-PCR was performed using SYBR Green I and its detection by LightCycler (Roche Molecular Biochemicals).
RESULTS

**PTEN**<sup>−/−</sup> bone marrow cells show increased osteoclastogenic capacity in vitro

To confirm the deletion efficiency of the conditional PTEN gene ablation we first analysed PTEN in OCs by immunoblotting. Therefore, we stimulated BMCs derived from PTEN<sup>fl/fl</sup> LysM cre mice (myeloid PTEN<sup>−/−</sup>) and littermate control wt mice with M-CSF and RANKL to generate OCs. Indeed, quantification revealed that deletion efficiency was more than 90% (figure 1A). Moreover, we analysed effects on the deletion on PI3K signalling in myeloid PTEN<sup>−/−</sup> OCs. As expected, we found the phosphorylation of downstream targets of PI3K such as AKT or GSK3β to be constitutively enhanced in OCs derived from myeloid PTEN<sup>−/−</sup> as compared with wt littermate controls (figure 1B).

We next investigated the osteoclastogenic potential of BMCs by evaluating the numbers of multinucleated TRAP<sup>+</sup> OCs and found a marked increase in OC numbers in cultures of BMCs derived from myeloid PTEN<sup>−/−</sup> compared with wt BMCs (figure 2A,B). Since the PI3K pathway is known to regulate proliferation and survival of cells, we investigated whether enhanced osteoclastogenesis was due to enhanced proliferation of osteoclast precursors (pOCs) after stimulation with M-CSF. However, we did not detect any differences between the numbers of pOCs under these conditions in the two groups (figure 2C). Additionally, when we labelled BMCs with carboxyfluorescein succinimidyl ester (CFSE) and measured CFSE dilution 3 days after stimulation with M-CSF, there was no difference between BMCs derived from myeloid PTEN<sup>−/−</sup> as compared to wt (not shown).

In line with these findings, analysis of apoptosis by AnnexinV/7-aminoactinomycin D (7AAD)-staining did not reveal differences in the amount of apoptotic pOCs from myeloid PTEN<sup>−/−</sup> as compared with wt (figure 2D). However, activation of PI3-kinase after stimulation with M-CSF is important for survival in wt as well as in myeloid PTEN<sup>−/−</sup>; as inhibition of this pathway with wortmannin led to increased proportions of AnnexinV/7AAD positive cells in both genotypes (see online supplementary figure S1). Taken together, these data indicate that the absence of PTEN has no effect on pOC proliferation or apoptosis.

We subsequently analysed the role of PTEN on survival of OCs. We found that osteoclastogenesis in BMCs from myeloid PTEN<sup>−/−</sup> and wt mice peaked 3 days after stimulation with RANKL with the number of OCs declining after this time point in similar fashion in both groups (figure 2E). However, osteoclastogenesis started earlier and the number of resulting OCs was higher when we used myeloid PTEN<sup>−/−</sup> BMCs to differentiate OCs. This suggests that enhanced osteoclastogenesis was due to enhanced RANKL-mediated differentiation of OCs and not due to enhanced generation of pOCs or enhanced survival of differentiated OCs.

To analyse the molecular basis for enhanced RANKL-mediated differentiation of pOCs to OCs in myeloid PTEN<sup>−/−</sup> BMCs, we measured NFATc1 and indeed, detected a marked upregulation of NFATc1 in myeloid PTEN<sup>−/−</sup> BMCs compared with wt by qPCR as well as western blotting (figure 2F,G). We next analysed mRNA expression of other OC-related genes and did not find significant differences in the expression of calcitonin-receptor or the M-CSF-receptor macrophage colony-stimulating factor receptor/CD115 (cFMS) between myeloid PTEN<sup>−/−</sup> and wt BMCs. However, mRNA of several OC effector genes including MMP9, TRAP and cathepsin K, was significantly overexpressed in OCs derived from myeloid PTEN<sup>−/−</sup> cells compared with wt cells (figure 3A–E).

We also tested the capacity of myeloid PTEN<sup>−/−</sup> OCs to resorb bone. We found no difference between the two groups (figure 3F). Taken together, we found that PTEN is important in regulating osteoclastogenesis, but has no effect on OC function per se.

**Increased in vivo osteoclastogenesis in myeloid-specific PTEN<sup>−/−</sup> mice**

Next, we asked whether PTEN deficiency also affects in vivo osteoclastogenesis. To answer this question, we analysed histological sections of tibial bones from 16-week-old myeloid-specific PTEN<sup>−/−</sup> mice and wt mice. We found a significant increase in the number of OCs per bone perimeter (NOc/BPm) as well as OC surface per bone surface (OcS/BS) in myeloid-PTEN<sup>−/−</sup> mice compared with wt animals (figure 4A). In line with our in vitro data, this indicates that the absence of PTEN also enhances osteoclastogenesis in vivo.

PTEN deficiency in osteoblasts has been reported to increase bone formation via enhanced proliferation and function of osteoblasts. Therefore, we asked whether the absence of PTEN restricted only to myeloid cells, but not affecting mesenchymal osteoblasts has indirect effects on osteoblast function in vivo. Indeed, when we analysed the MAR, we detected a

![Figure 1](http://ard.bmj.com/2013-203486)
significant increase in myeloid PTEN<sup>−/−</sup> mice compared with wt mice (figure 4B).

We could not detect any differences between myeloid PTEN<sup>−/−</sup> mice and wt animals in bone volume per tissue volume (BV/TV), trabecular thickness (TbTh), trabecular number (TbN) or trabecular separation (TbSp), demonstrating an unchanged net balance of bone turnover (figure 4C).

Figure 3  Receptor activator of nuclear factor κB ligand -stimulated myeloid phosphatase and Tensin homolog (PTEN)<sup>−/−</sup> bone marrow cells display enhanced expression of osteoclast (OC)-related genes. (A–E) qPCR analysis of the indicated mRNAs. Plots show mean values±SD, and are representative of at least 3 independent experiments. (F) Pit resorption assay of OCs derived from wildtype and myeloid PTEN<sup>−/−</sup> mice. The total area of resorption was divided by the total number of OC, resulting in area of resorption per OC, using the Osteomeasure software. CathK, cathepsin K; cFMS, macrophage colony-stimulating factor receptor/CD115; MMP9, matrix metalloproteinase 9; TRAP, tartrate-resistant acid phosphatase.

Myeloid PTEN<sup>−/−</sup> mice suffer from severely reduced grip strength despite similar paw swelling, as compared with wt mice

We next evaluated whether there is an impact of PTEN deficiency in myeloid cells on arthritis development, especially with regard to local osteoclastogenesis and bone destruction. Therefore, we crossed hTNF transgenic (hTNFtg) mice, which
Increased osteoclastogenesis in vivo in myeloid phosphatase and tensin homolog (PTEN)$^{−/−}$ mice. (A) Bone histomorphometry of osteoclast-related bone parameters of wildtype (wt) and myeloid PTEN$^{−/−}$ mice (age: 16 weeks). Plots are mean values±SD (n=7) (*p≤0.05). (B) Quantitative expression of the mineral apposition rate in wt and myeloid PTEN$^{−/−}$ mice (age: 16 weeks) (*p≤0.05). (C) Bone histomorphometry of wt and myeloid PTEN$^{−/−}$ mice (age: 16 weeks). Plots are mean values±SD (n=7) (*p≤0.05).

**Figure 4**

DISCUSSION

In this study, we provide evidence for an important role of the PI3K/PTEN pathway in OCs as deficiency of PTEN, and subsequent sustained PI3K activity in myeloid cells including pOCs leads to increased osteoclastogenesis in vitro and in vivo.

Our data show that absence of PTEN in myeloid cells augments the potential of monocytic OC precursors to differentiate into OCs under various conditions. Although proliferation and survival of myeloid PTEN$^{−/−}$OCs is not altered under in vitro conditions, their RANKL induced differentiation into OCs is highly increased. This is accompanied by an increased induction of NFATc1, known to be a master regulator of the RANKL-RANK pathway. Of note, neither the life span nor the resorptive capacity of myeloid PTEN$^{−/−}$OCs is affected. This is also observed under homeostatic conditions in vivo, as OCs numbers are markedly increased in bones of myeloid PTEN$^{−/−}$ mice. However, these mice do not become osteopenic due to compensation by a hyperactivity of osteoblasts, as shown by the significant increase in bone apposition rates in myeloid PTEN$^{−/−}$ mice.

The data we obtained in our experiments are different from those reported for another inhibitor of PI3K, SHIP. SHIP-deficient animals are osteoporoic due to increased...
myeloid PTEN
wortmannin increased the number of apoptotic cells in wt and
myeloid membrane of hTNFtg and hTNFtg/myeloid PTEN−/−
mice. We could also con
macrophages have clearly shown that activation of PI3-kinase
tially inhibited by SHIP and PTEN. Experiments using human
observed in the bones comprising the involved joints of myeloid
flammation in the synovial membrane of hTNFtg (n=15)
and hTNFtg/myeloid PTEN−/− mice (n=11). (B) Mean intensity of nuclear
determines the survival of macrophages.34 We could also con
the hind paws of hTNFtg and hTNFtg/myeloid PTEN−/− mice (*p<0.05). (E) Analysis of the percentage of F4/80+
cells shown as percent of total cells in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. (f) Analysis of the percentage of Gr1+
cells shown as percent of total cells in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. (g) Analysis of the number of OCs per area of inflammation in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. (*p<0.05; **p<0.01; ***p<0.001). (H) Analysis of the area of erosion caused by OCs in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice.

osteooclasis and OC function.24 It should be noted, however, that the mice used in the above-mentioned study exhibited a constitutive and generalised SHIP deficiency, whereas in our setting, PTEN deficiency is conditional and selectively affects myeloid cells. Nevertheless, there seem to be specific differences in the signalling pathways that are differentially inhibited by SHIP and PTEN. Experiments using human macrophages have clearly shown that activation of PI3-kinase regulates the survival of macrophages.34 We could also confirm this finding, since the inhibition of PI3-kinase signalling by wortmannin increased the number of apoptotic cells in wt and myeloid PTEN−/− BMCs. However, deletion of PTEN does not lead to reduced apoptosis in these cells, a phenomenon that has also been noted in SHIP-deficient BMCs.25 Additionally, blood counts of monocytes and neutrophils, as well as the relative number of CD11b cells in the spleen were not different between wt and myeloid PTEN−/− mice (see online supplementary figure S2), arguing against a major proliferative or survival advantage of myeloid cells in myeloid PTEN−/− compared to wt. Potential effects of SHIP deficiency involving the mesenchymal cell compartment, which plays a role in bone biology, namely osteoblasts and osteocytes, could at least partly contribute to the reduced bone mass detected in these mice.

Interestingly, the importance of PTEN in terminal differentiation of OCs under inflammatory conditions can also be observed in the bones comprising the involved joints of myeloid PTEN−/− animals overexpressing hTNF. Although synovial inflammation is only insignificantly increased, hTNFtg/myeloid PTEN−/− mice suffer from considerably increased local bone destruction. This is associated with highly decreased grip strength scores of hTNFtg/myeloid PTEN−/− mice compared with hTNFtg animals. In line with the data obtained previously, the numbers of pOCs in the inflamed synovial membrane are not different from those in hTNFtg animals. The numbers of mature OCs, however, are markedly increased in hTNFtg/myeloid PTEN−/− mice. This argues for a specific role of the P3K/PTEN axis in RANKL/RANK-driven osteoclastogenesis by facilitating the generation of bone resorbing OCs from the OC precursor pool (see online supplementary figure S3). Given the importance of bone erosion for the loss of function in patients with RA we think that the discovery of mechanisms leading to bone erosions independent from inflammation is of great importance for the development of new therapeutic strategies. Although it is difficult to draw a direct translational conclusion based on these results alone, they nonetheless suggest that restricting P3K activity in myeloid cells might be useful in inhibiting local bone destruction in arthritis.

Correction notice This article has been corrected since it was published Online First. The funding section has been updated.

Acknowledgements We thank Tetyana Shvets, Aurica Jelinek for expert technical assistance. We thank Dr George Kollias for providing the hTNFtg mice.

Basic and translational research

Figure 6 Joint inflammation and joint destruction are dissociated in human TNF-transgenic (hTNFtg)/myeloid phosphatase and tensin homolog (PTEN)−/− mice. (A) Quantitative histomorphometric analysis of inflammation in the synovial membrane of hTNFtg (n=15) and hTNFtg/myeloid PTEN−/− mice (n=11). (B) Mean intensity of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) expression in osteoclasts (OCs) in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. Mean area of erosion (C) and number of OCs (D) in the tarsal area of the hind paws of hTNFtg and hTNFtg/myeloid PTEN−/− mice (*p<0.05). (E) Analysis of the percentage of F4/80+ cells shown as percent of total cells in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. (f) Analysis of the percentage of Gr1+ cells shown as percent of total cells in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. (g) Analysis of the number of OCs per area of inflammation in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice. (*p<0.05; **p<0.01; ***p<0.001). (H) Analysis of the area of erosion caused by OCs in the synovial membrane of hTNFtg and hTNFtg/myeloid PTEN−/− mice.

Contributors SB, GS, JSS and KR: designed the experiments. SB, MF, AP, TL, ES, VS, JB, PM, BN and GS: performed experiments. SB, AP and GS: analysed results. SB and GS: made the figures. SB, GS, JSS and KR wrote the paper.

Funding This research has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement number 115142 (BTCure), resources of which are composed of financial contribution from the European Union’s Seventh Framework Programme and EFPIA companies’ in kind contribution, by the Coordination Theme 1 (Health) of the European Community’s FP7, Grant Agreement number HEALTH-F2-2008-223404 (Masterswitch), and by a grant from the Austrian science fund (FWF), number P 23730.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES