Aminobisphosphonates (NBP) are commonly used for treatment of hypercalcemia, osteoporosis and metastatic bone disease. Frequently, patients undergoing NBP treatment experience side-effects such as flu-like symptoms and elevated body temperature, also known as an acute phase response (APR), resulting from cytokine production by phosphoantigen-activated $\gamma\delta$-T cells. As opposed to NBP, statins inhibit hydroxymethylglutaryl coenzyme-A reductase, which catalyzes the first committed step of the (phosphoantigen-producing) mevalonate pathway. Statins therefore reduce intracellular phosphoantigen levels, and can prevent NBP-induced $\gamma\delta$-T cell activation, at least in vitro. We conducted a pilot study in patients with (bone-)metastasized malignancies with an indication to receive treatment with NBP, and evaluated the effects of NBP treatment on the phenotype and function of circulating $\gamma\delta$-T cells in vivo and whether statins could diminish NBP-induced $\gamma\delta$-T cell responses and the associated APR. We observed that administration of NBP alone or in combination with simvastatin resulted in the frequent occurrence of APR. Although we observed a reduced expression of perforin, granzyme B and HLA-DR in circulating $\gamma\delta$-T cells in patients treated with the combination of NBP and statins, statin treatment could not prevent NBP-induced changes in circulating $\gamma\delta$-T cell numbers or reduce $\gamma\delta$-T cell production of IFN$\gamma$ and TNF$\alpha$ triggered by NBP. Consistent with this failure to suppress in vivo $\gamma\delta$-T cell responses, simvastatin could not fully prevent the occurrence of APR upon NBP-infusion. These observations call for the exploration of alternative strategies aimed at a more direct inhibition of $\gamma\delta$-T cell activation to prevent APR as a collateral of NBP treatment.
minobisphosphonates (NBP, e.g. pamidronate and zoledronic acid) are frequently used for the treatment of hypercalcemia, osteoporosis and metastatic bone disease of both hematopoietic (e.g. multiple myeloma) and non-hematopoietic (e.g. breast and prostate cancer) malignancies [1]. NBP exposure results in a defective regulation of processes in osteoclasts, due to inhibition of a crucial step in the mevalonate pathway, an important metabolic pathway of which cholesterol is a well-known product [2,3]. Approximately one third to half of all patients undergoing NBP treatment experience side effects such as flu-like symptoms (chills, fatigue, myalgia) and elevated body temperature, also known as an acute phase response (APR) [4,5]. It has been proposed that APR results from cytokine production by $V_\gamma V_\delta$-T cells [6,7].

$V_\gamma V_\delta$-T cells are the predominant $\gamma\delta$-T cell subset in human peripheral blood and are considered innate-like effector cells. They recognize a restricted set of phosphorylated compounds, referred to as phosphoantigens (pAg) and are involved in a variety of processes related to anti-microbial- and anti-tumor immunity. $V_\gamma V_\delta$-T cells can be directly activated by natural and synthetic pAg (e.g. IPP, BrHPP, and HMBPP), whereas other compounds such as NBP, sensitize target cells to $V_\gamma V_\delta$-T cell recognition by stimulating intracellular accumulation of endogenous pAg through inhibition of the enzyme farnesyl diphosphate (FPP) synthase in the mevalonate pathway [8–11], inducing conformational changes in CD277/butyrophilin-3A (BTN3A) molecules and cognate triggering of $V_\gamma V_\delta$-T cell activation and expansion [12]. As opposed to NBP, statins inhibit hydroxymethylglutaryl coenzyme A reductase, which catalyzes the first committed step of the mevalonate pathway. Therefore, statins, drugs that are principally used to regulate hypercholesterolemia, induce diminished intracellular IPP levels, and can thereby prevent the NBP-induced $V_\gamma V_\delta$-T cell activation, as demonstrated in vitro [7,13].

A correlation between the activation status of $V_\gamma V_\delta$-T cells and APR incidence upon NBP stimulation was initially observed by Kunzmann et al., reporting that all of the patients that developed APR showed a substantial increase in the number of circulating $V_\gamma V_\delta$-T cells when measured 1 and 3 weeks post-infusion [5]. The adverse effects in response to NBP treatment can be severe enough for patients to decline further therapy [14], underlining the need for a therapeutic agent to mitigate the occurrence of this clinical response. However, the severity of clinical APR upon repeated administration of NBP appears to diminish over time [5], suggesting that $V_\gamma V_\delta$-T cells, as the responsible cells for the APR, become anergic or exhausted upon repeated encounter with activation stimuli. At least two clinical studies attempted to prevent the development of
The effects of systemic treatment with aminobisphosphonates and statins on circulating Vγ9Vδ2-T cells in patients with advanced cancer

APR by co-treatment with statins. Both studies reported no effect of statins (atorvastatin [14] and fluvastatin[15]) on the occurrence of APR. Statins were administered one day before or at the time of intravenous NBP administration. Possibly, prolonged treatment with statins before NBP infusion might have resulted in enhanced immunological and clinical efficacy.

With the aim to evaluate the inhibitory effects of statins on NBP-induced activation of circulating Vγ9Vδ2-T cells in vivo, we conducted a pilot study in patients with (bone-)metastasized malignancies who had an indication to receive i.v. treatment with NBP, and set out to evaluate 1) the effects of NBP treatment on the phenotype and function of circulating Vγ9Vδ2-T cells in vivo and 2) whether statins could diminish NBP-induced Vγ9Vδ2-T cell responses and collateral clinical APR. In contrast to previous studies [14,15], we assessed the impact of orally administered statins, starting at least 1 week prior to NBP treatment, in order to ensure a more profound inhibition of the mevalonate pathway prior to NBP infusion. Furthermore, where other studies focused mainly on numbers of lymphocyte subsets, we additionally also assessed various phenotypic and functional Vγ9Vδ2-T cell parameters in response to NBP infusion.

Patients, materials and methods

Study population
This single center study was performed in the Department of Medical Oncology of the VU University Medical Center (Amsterdam, The Netherlands) between November 2010 and January 2013 and included seventeen adult patients with hypercalcemia and/or bone metastases from various types of (solid) cancers (including breast cancer n=9, prostate cancer n=2, esophageal cancer n=2, urothelial cell carcinoma n=1, HNSCC n=1, renal cell carcinoma n=1, salivary gland cancer n=1) with an indication to be treated with the i.v. administered NBPs pamidronate or zoledronic acid. The mean age of patients was 65 ± 11 (± SD) years (range, 40–88 years), with 8 males and 9 females. There was no unbalance between the statin (n=5) vs non-statin group (n=12), concerning type of disease or age (statin group 62 ± 8, non-statin group 67 ± 12; mean (yr) ± SD). All patients signed informed consent according to guidelines of the local medical ethics committee. Eligibility criteria included (1) a World Health Organization (WHO) performance score of less than 3 and (2) no severe impairment of renal or hepatic function. Main exclusion criteria included previous treatment with NBP, simultaneous use of immunosuppressive medication or CYP3A4 inhibitors,
Patients already using statins continued statin treatment, while the other patients were randomized to receive NBP alone or NBP in combination with simvastatin (40 mg once daily), initiated one week prior to the administration of NBP and to be continued until the following i.v. NBP administration. Of the 17 included patients, 5 (29.4%) patients received statin therapy concurrent with NBP. Patient evaluation and blood examination was performed at four points in time: at baseline (T1), day 1 (T2), day 7 (T3), and day 21 or 28 (T4) depending on the 3- or 4-weekly NBP treatment schedule that was planned by the treating physician.

Drug Formulation and Administration
Pamidronate disodium 3 mg/ml concentrate solution for infusion was administered at 60 mg (3-weekly, 23.5% of patients) or 90 mg (4-weekly, 70.6% of patients) in 250 ml NaCl 0.9%. One patient (5.9% of patients) received 4 mg zoledronic acid in 250 ml NaCl 0.9%. We did not find differences in our objectives between doses or type of bisphosphonate.

Treatment toxicity
Toxicity and WHO performance scores were assessed before treatment (T1), and at days 1 (T2), 7 (T3) and either day 21 or 28 (T4). Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0. Before treatment and during the first 2 days following NBP administration, the body temperature was measured by the participants 3 times daily.

Immune monitoring
To assess the biological activities of NBP and statins, immune monitoring was performed. For this purpose approximately 20 ml of heparinized PB was collected before the first administration (T1), and on days 1 (T2), 7 (T3) and 21 or 28 (T4). Time point 4 was the last measurement before the next administration of NBP.

Preparation of peripheral blood mononuclear cells (PBMC) for cryopreservation
PBMC were isolated from heparinized diluted peripheral blood by Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. Cells were washed and suspended at 4°C in IMDM (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Amsterdam, The Netherlands), 100 I.E./
ml sodium penicillin (Astellas Pharma, Leiden, The Netherlands), 100 μg/ml streptomycin sulphate (Radiumfarma-Fisiopharma, Naples, Italy), 2.0 mM MB-glutamine (Life Technologies, Bleiswijk, The Netherlands) and 0.05 mM 2-mercaptoethanol (Merck, Darmstadt, Germany). Cells were stained immediately or were cryopreserved and stored in liquid nitrogen until use, allowing for simultaneous functional testing of all of the PBMC samples from each individual patient. There were no differences observed for the monitored immune subsets in the results between these procedures.

**Flow-cytometry**

The antibodies used for flow cytometry analysis of PBMC were Fluorescein isothiocyanate (FITC)-, Phycoerythrin (PE)-, Peridinin Chlorophyll Protein (PerCP)- or Allophycocyanin (APC)-labeled mAbs directed against CD3, CD4, CD8, CD25, CD69, CD80, CD86, HLA-DR (BD Biosciences, New Jersey, USA), CD40, CD83 (Immunotech, Marseille, France), TCR-Vδ2 (BD Biosciences (PerCP) and Beckman Coulter, Inc. (FITC), TCR-Vγ9 (Biolegend, San Diego, USA (PE and AP)), CD27 and CD45RA (both BD Biosciences). mAb staining was performed in PBS supplemented with 0.1% BSA and 0.02% sodium-azide (NaN₃) for 30 min at 4°C. All stained cells were analyzed on FACS Calibur (BD Biosciences) using CellQuest software. Absolute numbers of each leukocyte subset at all four time points were obtained from whole-blood samples. Measurement of absolute numbers of leucocytes was performed using the Casy counter (Schärfe System). For analyses of absolute and relative numbers of CD14, CD3, and CD19 positive cells whole-blood was stained for flowcytometry in Trucount Tubes, and analyzed using a lyse-no-wash procedure on a FACS Calibur (BD Biosciences).

For intracellular flow cytometry, PBMC were placed in a 96-well round-bottom plate (200 µl) in the presence of 0.5 µl of brefeldin A (GolgI Plug, BD Biosciences). After 5 h, cells were harvested, washed and stained with PerCP- or APC-labeled Abs directed against human Vδ2 and Vγ9. After fixation with Cytotix/Cytoperm solution and permeabilization with Perm/Wash solution (BD Biosciences), cells were stained with mAbs specific for IFNγ or TNFα (BD Pharmingen). To determine granzyme B and perforin expression, permeabilized cells were stained with anti-human granzyme B (Pelicluster, Sanquin, Amsterdam, The Netherlands), anti-human perforin (eBioscience) or the appropriate isotype controls. All stained cells were analyzed on a FACS Calibur (BD Biosciences) using CellQuest software.
Statistical analysis
All data were analysed using one-way Anova, in combination with Dunnett or Dunns post-hoc test as considered appropriate. Findings were considered statistically significant when p-values were \( \leq 0.05 \), as indicated with asterisks (* \( p \leq 0.05 \), ** \( p \leq 0.01 \), *** \( p \leq 0.001 \)). Statistical analyses were performed using GraphPad Prism software (version 5.02, 2008).

Results

Patient characteristics
Seventeen patients were enrolled in this study, with a mean age of 65 ± 11 years (± SD) (range 40–88 years). All patients were diagnosed with advanced-stage cancer and received NBP as palliative therapy in case of bone metastases and/or hypercalcemia. Patients enrolled in the concurrent statin treatment arm were patients that already used statin for cardiovascular indications \( (n=3) \) or were allocated to the statin treatment arm of the study receiving simvastatin 40 mg once daily, starting 1 week prior to the administration of NBP and continued for a maximum of 5 weeks.

Relation between phenotypic and functional changes in Vγ9Vδ2-T cells and the occurrence of APR
In our study 8 patients (47.1%) developed transient APR with (low-grade) fever (defined as a body temperature ≥ 37.5 °C in combination with an increase of ≥ 0.5 °C after NBP administration) and/or chills (grade 1-2) following NBP therapy. This effect was most prominent in the first two days after NBP administration. Myalgia occurred in 4 out of these 8 patients (50%). Three of five (60%) patients using concurrent statins developed APR upon NBP infusion. Interestingly, all patients but one (94.1%) already used acetaminophen (paracetamol) for pain-relief, a drug well known for its analgetic and antipyretic function. As half (47.1%) of our study group developed APR after NBP infusion, even under the concurrent use of acetaminophen, this underscores the frequency and severity of this response following NBP administration.

A correlation between the development of APR and an increase in the frequency of circulating Vγ9Vδ2-T cells has been reported by Kunzmann et al [5,8]. We similarly found this increase in Vγ9Vδ2-T cell rates after an initial drop one day after NBP infusion to be most pronounced and to reach statistical significance only in patients with symptoms compatible with APR (Fig. 1).
Moreover, with the exception of CD69, perforin and TNFα (Fig. 1) where NBP-induced changes were consistently observed regardless of the occurrence of APR, the observed increases in activation markers and effector molecules in Vy9Vδ2-T cells following NBP administration were more pronounced and significant in patients experiencing APR. Also, the observed increases in CD45RA+/CD27- effector Vy9Vδ2-T cells were restricted to patients experiencing APR (Fig. 1). From these observations we conclude that APR is indeed related to the induction of type-1 effector Vy9Vδ2-T cells.

Figure 1. Effect of aminobisphosphonates on Vy9Vδ2-T cell activation in patients with an acute phase response (APR). The effect of systemic NBP treatment on the Vy9Vδ2-T cell frequency, their expression of CD69, perforin, granzyme B, Th1 cytokines IFNγ and TNFα, the surface molecules HLA-DR and CD86, and on the effector phenotype (CD45RA+/CD27-) of Vy9Vδ2-T cells, in relation to the occurrence of APR-related side effects. Shown are relative means of percentages ± SEM of marker expression on Vy9Vδ2-T cells in APR positive (dark grey bars) or APR negative (light grey bars) patients compared to T0 (set as 1). The mean frequency of marker expression as percentages of Vy9Vδ2-T cells at baseline (T0) are listed.

**On-treatment Vy9Vδ2-T cell rates**

NBP administration induced no significant changes in absolute numbers of leucocytes or lymphocytes. A moderate, but significant, decrease in percentages of CD3+ T cells (as % of lymphocytes) was found one week after NBP treatment in the group of patients receiving statins (Fig 2A). Pan-γδ-T cells, as a percentage of lymphocytes, significantly increased after 7 days (T3) compared to the transient decrease 1 day after NBP infusion (Fig 2B). This appeared mainly due to changes
Figure 2. Effect of aminobisphosphonates on CD3+ T cells, pan γδ-T cells and Vγ9Vδ2-T cells. Effect of NBP or combined NBP and statin administration on the frequency of CD3+ T cells (A), pan γδ-T cells (B) and Vγ9Vδ2-T cells (C) of lymphocytes. Shown are relative means ± SEM of marker expression in all patients (grey bars) and in patients using statins (black bars) or no statins (white bars) compared to T0 (set as 1). Also the mean frequency of these cells expressed as percentages of lymphocytes are listed.
The effects of systemic treatment with aminobisphosphonates and statins on circulating $\gamma\delta$ T cells in patients with advanced cancer

in the predominant $\gamma\delta$-T cell subset as the $\gamma\delta$-T cell rates similarly significantly dropped after NBP infusion and increased significantly over T2 levels at day 7 (Fig 2C). Of note, such a rebound effect with increased levels of $\gamma\delta$-T cells at T3 over T2, was not observed in patients concurrently receiving statins.

**On-treatment phenotypic and functional alterations in $\gamma\delta$-T cells**

Next, we evaluated the effect of systemic NBP treatment on activation of $\gamma\delta$-T cells. In order to evaluate $\gamma\delta$-T cell specific effects (Fig. 3A), non-$\gamma\delta$ cells ($\gamma$ and $\delta$ double negative lymphocytes) were also assessed as a control population (Fig. 3B). After NBP infusion at baseline (T1) a significant increase in the expression of the early activation marker CD69 by $\gamma\delta$-T cells (but not by non-$\gamma\delta$-lymphocytes [Fig.3B]) was found at T2, which dropped back to baseline levels after 4 weeks (T4) (Fig 3A). When divided in statin vs non-statin groups trends were similar, but did not reach statistical significance in patients receiving statins (Fig 3C). Similarly, a significant and specific transient up regulation of the effector molecules perforin, IFNg, and TNFα was observed in and restricted to $\gamma\delta$-T cells (Fig. 3A and 3B). Of note, of these only the levels of perforin were profoundly attenuated by concurrent statin administration (Fig. 3C). Interestingly, differential NBP-induced expression kinetics were observed for the effector molecule granzyme B. Significant and specific increases in granzyme B expression by $\gamma\delta$-T cells occurred one week after NBP infusion and persisted until the following NBP infusion (T4, Fig. 3A). In statin-treated patients a similar but dampened granzyme B response was observed with post-NBP increases failing to reach statistical significance (Fig. 3C).

**On-treatment expression of APC markers in $\gamma\delta$-T cells**

$\gamma\delta$-T cells have previously been shown to acquire professional Ag presenting capacities upon their activation by pAg [16–19]. To determine whether such an acquisition of APC characteristics also occurred after in vivo activation of $\gamma\delta$-T cells by NBP, we monitored expression levels of CD40, CD80, CD83, CD86, and HLA-DR on $\gamma\delta$-T cells after NBP stimulation. Whereas HLA-DR and CD86 levels were upregulated by day 7 in NBP-treated patients without concurrent statin administration (T3, Fig. 3C), no clear and consistent effects on CD40, CD80 and CD83 levels were observed (data not shown). From this, we conclude that the upregulated HLA-DR and CD86 levels most likely result from
Figure 3. Effect of aminobisphosphonates on Vy9Vδ2-T cell activation. Expression of CD69, perforin, granzyme B and Th1 cytokines IFNγ and TNFα by Vy9Vδ2-T cells (A) and Vy9-Vδ2- cells (B) upon systemic NBP treatment. Shown are relative means of percentages of marker expression ± SEM by Vy9Vδ2-T cells and Vy9-Vδ2- cells. Fig 3C. additionally depicts expression of the surface molecules HLA-DR and CD86 by Vy9Vδ2-T cells of patients concurrently using statins (black bars) or not using statins (white bars) with systemic NBP treatment. Shown are relative means of percentages of marker expression ± SEM by Vy9Vδ2-T cells compared to T0 (set as 1). The mean frequency of marker expression as percentages of Vy9Vδ2-T cells and Vy9-Vδ2- cells at baseline (T0) are listed.
general T cell activation [20] without acquisition of APC capabilities, which would have required concerted co-expression induction of additional APC-related markers such as CD40, CD80 and CD83.

**On-treatment changes in effector memory phenotype of Vγ9Vδ2-T cells**
In analogy to CD8⁺ T cells, the following differentiation states of Vγ9Vδ2-T cells were discerned based on surface expression of CD27 and CD45RA: effector T cells (CD45RA⁺CD27⁻), naive T cells (CD45RA⁺CD27⁺), effector memory T cells (CD45RA⁻CD27⁻), and central memory T cells (CD45RA⁻CD27⁺). Before start of treatment, Vγ9Vδ2-T cells showed mainly a central memory phenotype as compared to non-Vγ9Vδ2-cells (Vγ9Vδ2-T cells vs non-Vγ9Vδ2-cells 47.4 ± 5.7 vs 31.3 ± 7.6, p < 0.05). As shown in Fig. 4 (upper panel), administration of NBP led to a significant transient increase in the frequency of Vγ9Vδ2-T cells with an effector phenotype one day after systemic administration of NBP. In contrast, conventional effector T cells showed only a very modest (though significant) increase one week after NBP administration (Fig. 4, lower panel). Of note, concurrent administration of statins did not affect these changes in effector Vγ9Vδ2-T cells (data not shown).

**Discussion**
Here, we set out to determine the effects of systemic administration of NBP on the frequency, phenotype, and activation status of circulating Vγ9Vδ2-T cells and to evaluate whether these effects, as well as the occurrence of the NBP induced APR, could be prevented or counteracted by the concurrent oral use of simvastatin.

Though a relatively small and transient decrease in peripheral blood T cells was noted between baseline and day 7 after NBP infusion, confirming an earlier observation from Adami et al. [4], a striking reduction in Vγ9Vδ2-T cell frequencies was found within the first day after NBP infusion. This rapid reduction in circulating Vγ9Vδ2-T cell numbers could be the result of an activation-induced adhesion to and/or transmigration of Vγ9Vδ2-T cells through the endothelial layer or, alternatively, result from down regulation and internalization of the Vγ9Vδ2 T cell receptor upon stimulation [21]. Vγ9Vδ2-T cell rates were fully restored, or even increased, in the peripheral blood seven days after NBP infusion. Frequently, Vγ9Vδ2-T cell numbers were found to increase to higher than pretreatment values, compatible with an expansion of this cell population,
Figure 4. The effect of aminobisphosphonates on the effector memory phenotype of Vy9Vδ2-T cells. The effector memory phenotype of A) Vy9Vδ2-T cells and B) Vy9Vδ2- cells at different time-points after systemic NBP infusion. Shown are relative means of percentages ± SEM of combinations of CD45RA and CD27 marker expression compared to T0 (set as 1). The mean frequency of combined marker expression as percentages of Vy9Vδ2-T cells and Vy9Vδ2- cells at baseline (T0) are listed. The following phenotypes are distinguished: CD45+CD27- effector T cells, CD45+CD27+ naive T cells, CD45+CD27- effector memory T cells, and CD45+CD27+ central memory T cells.

but these changes did not reach statistical significance. In most patients the percentage of circulating Vy9Vδ2-T cells returned to baseline levels before the second NBP infusion. These striking changes in the Vy9Vδ2-T cell population were not observed in conventional (non-Vy9Vδ2) lymphocytes, confirming the
selective effect of NBP on Vy9Vδ2-T cells.

The age and advanced cancer stage of our studied patient group may have attenuated the observed NBP-induced activation and expansion of Vy9Vδ2-T cells. In our study group the mean percentage of Vy9Vδ2-T cells of lymphocytes and of CD3+ T cells ± SEM was 0.87 ± 0.20 % and 1.41 ± 0.40 % respectively. The mean percentage of pan γδ-T cells of lymphocytes and of CD3+ T cells ± SEM was 1.94 ± 0.32 % and 3.26 ± 0.48 % respectively. These levels are substantially lower than levels reported in other studies where children or adults with non-malignant diseases are evaluated [13,14]. This notion is supported by the observation in our study that one female patient, who was relatively young and who was recently diagnosed with metastatic disease, showed a massive expansion of Vy9Vδ2-T cells (as a percentage of lymphocytes) from 2.9% to 14.9% one week post-infusion (T3), after an initial decrease in Vy9Vδ2-T cell levels to 0.5% of lymphocytes (T2). Importantly, 87.5% of APR patients demonstrated an increase in the amount of Vy9Vδ2-T cells compared to baseline level 1 week post-infusion, compared to 22.2% of patients without an APR (data not shown), showing that, even in this immune-challenged patient population, APR is related to expansion of circulating Vy9Vδ2-T cells.

Our data support in vitro findings that NBP induces selective activation of Vy9Vδ2-T cells [8]. Also, these results are in line with previous in vivo studies showing that Vy9Vδ2-T cells display mainly a central memory phenotype with a low proportion of naïve cells, as well as a transient increase in effector rates after pAg stimulation [22,23]. Moreover, these rapid and transient increases in effector rates are mirrored by similar increases in effector molecules like IFNγ, TNFα, granzyme B and perforin, supporting the notion that in vivo treatment with NBP induces Vy9Vδ2-T cells to mature towards a Th1 cytokine producing effector phenotype, possibly inducing more effective antitumor responses.

Overall, the administration of NBP alone or in combination with simvastatin resulted in a frequent occurrence of APR-related side effects. Approximately 47% of patients developed APR within the first two days after infusion of NBP. We set out to evaluate whether the effects of NBP on Vy9Vδ2-T cell responses and the APR could be prevented by the frequently prescribed cholesterol lowering drug simvastatin. Though we observed a diminished expression of perforin, granzyme B and the activation-related cell surface marker HLA-DR on Vy9Vδ2-T cells upon NBP administration in combination with statins, simvastatin treatment was not able to prevent the increase in Vy9Vδ2-T cell rates, nor was it capable of reducing the production of IFNγ and TNFα by Vy9Vδ2-T cells. In line with this failure to suppress Vy9Vδ2-T cell
responses, simvastatin was not able to prevent the occurrence of APR upon NBP-infusion. These data are in line with previous reports that statins failed to prevent NBP-induced in vivo APR [14,15]. Previous reports of Hewitt et al. and Thompson et al. have demonstrated that NBP-induced activation of Vγ9Vδ2-T cells was inhibited in vitro by both simvastatin and fluvastatin at a concentration of 100 nM [7,13]. This dose is found to be lower than the clinically relevant concentration of fluvastatin (>1 µM) and similar to the in vivo concentration of simvastatin (approximately 80 nM) [24]. In vivo these effects were studied by Thompson et al [15], who assessed clinical and hematological effects in patients after NBP administration in the presence or absence of concurrent fluvastatin administration, and Srivastava et al [14], who evaluated comparable responses in children receiving bisphosphonates in the presence or absence of concurrent atorvastatin administration. Both reports showed no effect of statins on the APR. However, statins were started the day before or the day of intravenous bisphosphonate therapy. In our study we started statin treatment at least 7 days before NBP-infusion to make sure that the oral administration route did not affect the biological availability, based on the observation that dose levels of statins in previous studies did not lead to sufficient inhibition of the mevalonate pathway in order to inhibit IPP production in response to NBP infusion. However, our present data show that the employed dose and administration schedule of statins does not suffice for the future prevention or treatment of APR-related side-effects after NBP infusion. Moreover, our data confirm observations from another group that became available during the conduct of our trial, in which Makras et al. evaluated clinical responses in relation to immune effects upon NBP treatment. In this paper it was reported that administration of rosuvastatin, started 5 days before infusion of zoledronic acid, did not prevent the occurrence of APR in osteoporotic women [25].

In conclusion, in vivo studies do not support a clinically relevant role of statins, at least not at doses commonly used for the treatment of hypercholesterolemia, in the prevention of APR upon NBP administration. More research should be done in order to explore new strategies that can prevent or counteract APR upon NBP treatment. Generally, patients are treated with best supportive care (e.g. acetaminophen) when APR develops, which led to the suggestion of treating all patients with acetaminophen when receiving NBP for the first time [26]. We demonstrated that even though the vast majority of our patients used acetaminophen, still 47.1% of patients developed APR, indicating the limitations of such an approach. Possibly, new strategies aimed at a more direct inhibition of Vγ9Vδ2-T cell cell activation upon NBP stimulation could be more
The effects of systemic treatment with aminobisphosphonates and statins on circulating Vγ9Vδ2-T cells in patients with advanced cancer

successful, e.g. by the use of blocking antibodies against the Vγ9Vδ2-TCR or against its activating ligand CD277/BTN3A1.

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The effects of systemic treatment with aminobisphosphonates and statins on circulating Vγ9Vδ2-T cells in patients with advanced cancer