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EFFICACY AND SAFETY OF γδ T CELL-BASED TUMOR IMMUNOTHERAPY: A META-ANALYSIS

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Vγ9Vδ2 T cells are important effector cells that may play a role in the anti-tumor immune response. Their capability to exert MHC-nonrestricted lytic activity against different tumor cells *in vitro* and their detection among tumor infiltrating lymphocytes in a variety of human cancers have supported the development of Vγ9Vδ2 T cell-based immunotherapy in the context of novel treatment against cancer. Accordingly, promising reports from recent clinical trials support the use of Vγ9Vδ2 T cells as immunotherapeutic agents, either via adoptive transfer of *ex-vivo* expanded Vγ9Vδ2 T cells or *in vivo* activation of Vγ9Vδ2 T cells with compounds such as phosphoantigens or aminobisphosphonates. In this study we have performed a meta-analysis to assess the objective efficacy and safety of Vγ9Vδ2 T cell-based immunotherapy. Database including Pubmed, Web of Science and SCOPUS were investigated to identify relevant studies. Thirteen clinical trials involving patients with advanced or metastatic cancer were selected. In order to estimate the strength of association between Vγ9Vδ2 T cell-based immunotherapy and favorable clinical effect or toxicity grade we used event rate (ER) with 95% confidence interval (CI). The total effective rate provided significant results (ER = 0.407; $P < 0.014$) while no correlation was found between serious adverse effects and Vγ9Vδ2 T cell-based therapy. This meta-analysis demonstrates that Vγ9Vδ2 T cell-based immunotherapy improves overall survival and, in view of its low toxicity grade, provides a proof of principle for its utilization as adjuvant to conventional therapies for resistant/refractory patients care.

Human γδ T lymphocytes comprise two main subsets defined by the T cell receptor (TCR): one subset expresses the Vδ1 chain paired to any Vγ chain and is localized in peripheral tissues, while the other subset expresses the Vδ2 chain preferentially paired to the Vγ9 chain (here and after referred to as Vγ9Vδ2 T cells) and predominate in lymphoid organs and peripheral blood (1-2).

Vγ9Vδ2 T cell activation is achieved by recognition, in non-major histocompatibility complex (MHC)-restricted fashion, of non-peptidic phosphorylated molecules, known as phosphoantigens (PAgs), produced through the isoprenoid biosynthesis pathways (3-5). PAgs are

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not stimulatory at physiologic levels, but tumor and infected cells, produce elevated levels of PAgs that are able to activate Vγ9Vδ2 T cells (6-8). Vγ9Vδ2T cells can also be activated, through an indirect mechanism, by aminobisphosphonates (N-BPs), a class of drugs used to treat bone metastases, that inhibit farnesyl pyrophosphate synthase and cause accumulation of endogenous upstream PAgs.

There are several direct and indirect evidences which support the clinical use of Vγ9Vδ2 T cells in cancer immunotherapy; a): Vγ9Vδ2 T cells perform non-MHC restricted cytotoxic activity against a broad variety of cancer cells and produce cytokines with known anti-tumor activity (9, 10); b): the localization of γδ T cells within epithelia and their capacity to infiltrate tumors suggest that these cells may contribute to the surveillance of malignancies (1, 2, 9); c): tumor-infiltrating Vγ9Vδ2 T cells have been found in a broad spectrum of malignancies although their prognostic value is controversial (11-14). Moreover, when autologous radiolabeled Vγ9Vδ2 T cells were injected into patients with advanced solid tumors, they localized predominantly in the lungs, liver, spleen and to sites of metastasis (15).

Altogether, these findings have clearly shown that Vγ9Vδ2 T cells constitute an important component of immune responses against tumors. Accordingly, several small clinical trials involving patients with advanced disease, refractory to conventional treatments, have been performed to assay the safety and efficacy of Vγ9Vδ2 T cells activated *in vivo* with PAgs/IL-2 or N-BPs/IL-2 and of the adoptive transfer of *ex vivo* preactivated autologous Vγ9Vδ2 T lymphocytes (15-27). However, the heterogeneity of therapeutic algorithms, non standardized cellular products and the lack of established criteria for clinical responses have made it impossible to draw valid conclusions from single clinical trials.

In this study, we used a meta-analysis based on data from pooled patient samples to obtain a more powerful estimate of the objective efficacy and side effects of Vγ9Vδ2 T cell-based immunotherapy in patients with advanced or metastatic chemotherapy-resistant tumors.

MATERIALS AND METHODS

Literature search strategy

Cancer clinical trials involving Vγ9Vδ2 T lymphocytes and performed from January 2000 to October 2012, were identified through a search on PubMed, Web of Science, and SCOPUS using the following keywords: "gammadelta T cell" or "gamma delta T cell" and "cancer immunotherapy" or "tumor immunotherapy". The search was performed in accordance to the relevant criteria from the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement. All references cited in these studies and published reviews were examined in order to identify additional works. To avoid duplication of data the body of each publication and the names of all authors were examined. The selection criteria used for the search included: 1): studies written in the English language and limited to human trials; 2): removal of case reports, reviews, comparative studies, opinion, pharmacologic and methodological articles etc; 3); studies adopting randomized and non-randomized groups including patients in advanced or metastatic stage.

Data extraction

Data extraction was carried out by one reviewer (SB) and independently checked for accuracy by a second reviewer (GG). When any discrepancy occurred the consensus was reached by discussion among the investigators. For each study, the following data were extracted: first author's surname, year of publication, clinical study phase, diagnosis criterion, stage of patients, number of patients, treatment design, dosage and toxicity.

The clinical outcomes used to evaluate effectiveness and safety of Vγ9Vδ2 T cell-based immunotherapy in advanced or metastatic tumors were progression disease (PD), stable disease (SD), partial response (PR) and complete response (CR), defined based on Response Evaluation Criteria in Solid Tumors (RECIST), with the exception of the antitumor effects reported by Kobayashi et al. (22), which were analyzed according to tumor-doubling time (DT). We assessed the objective immunotherapeutic response as event rate (SD+PR+CR/N. of patients).

All clinical studies included in this meta-analysis presented different adverse side effects (AEs) which were graded using the National Cancer Institute Common Toxicity Criteria, version 2.0 and 3.0 (31) except AEs reported by Wilhelm et al. (16), that instead were analyzed using the World Health Organization (WHO) criteria. We evaluated toxicity by classifying side effects into three groups: 1): mild; 2): moderate; 3); severe; and considering adverse events described in the works as follows: grade 1 plus 2 belonging to the first group, grade 3 to the second and grade 4 to the third one.

Statistical analysis

The meta-analysis was performed by using Comprehensive Meta Analysis V2. We used as effect sizes the event rate reported in each studies measured as

the number of patients showing a response to the therapy over the total number of treated patients. Testing the homogeneity of the effect sizes is an important issue when meta-analysis are performed since if the effect sizes are heterogeneous, the calculated Standard Errors (SEs) could be underestimated. In this context a simple approach relies on t^2 indicating the variability on the effect sizes which is larger than the sampling error. Despite t^2 cannot be interpreted directly, Higgins and Thompson (28) proposed several indices to quantify the degree of heterogeneity. One of them is the I^2 index. I^2 statistics is interpreted as the proportion of total variation contributed by between-study variation. If there was no statistical heterogeneity among the studies (I^2<50% and P>0.05), the event rate and 95% confidence interval (CI) would be estimated for each study in a fixed-effects model. Otherwise, a random-effect model should be employed. In our analysis we exploited the I^2 statistics to test heterogeneity among studies. In sensitivity analysis, relative influence of each study on the pooled estimate was assessed by omitting one study at a time. Funnel plots were used to evaluate publication and/or bias. Forest plots were supplemented with the overall Mantel-Haenszel estimate (fixed effect). All *P*-values were two-tailed.

RESULTS

Characteristic of articles in our meta-analysis

A total of 165 studies were identified by the searches. By scanning titles and abstracts, redundant publications, reviews, letters, opinion articles and control or case reports were excluded. After referring to full texts, we removed 152 studies that did not meet the selection criteria. As a result, 13 studies that included a total of 204 patients were selected

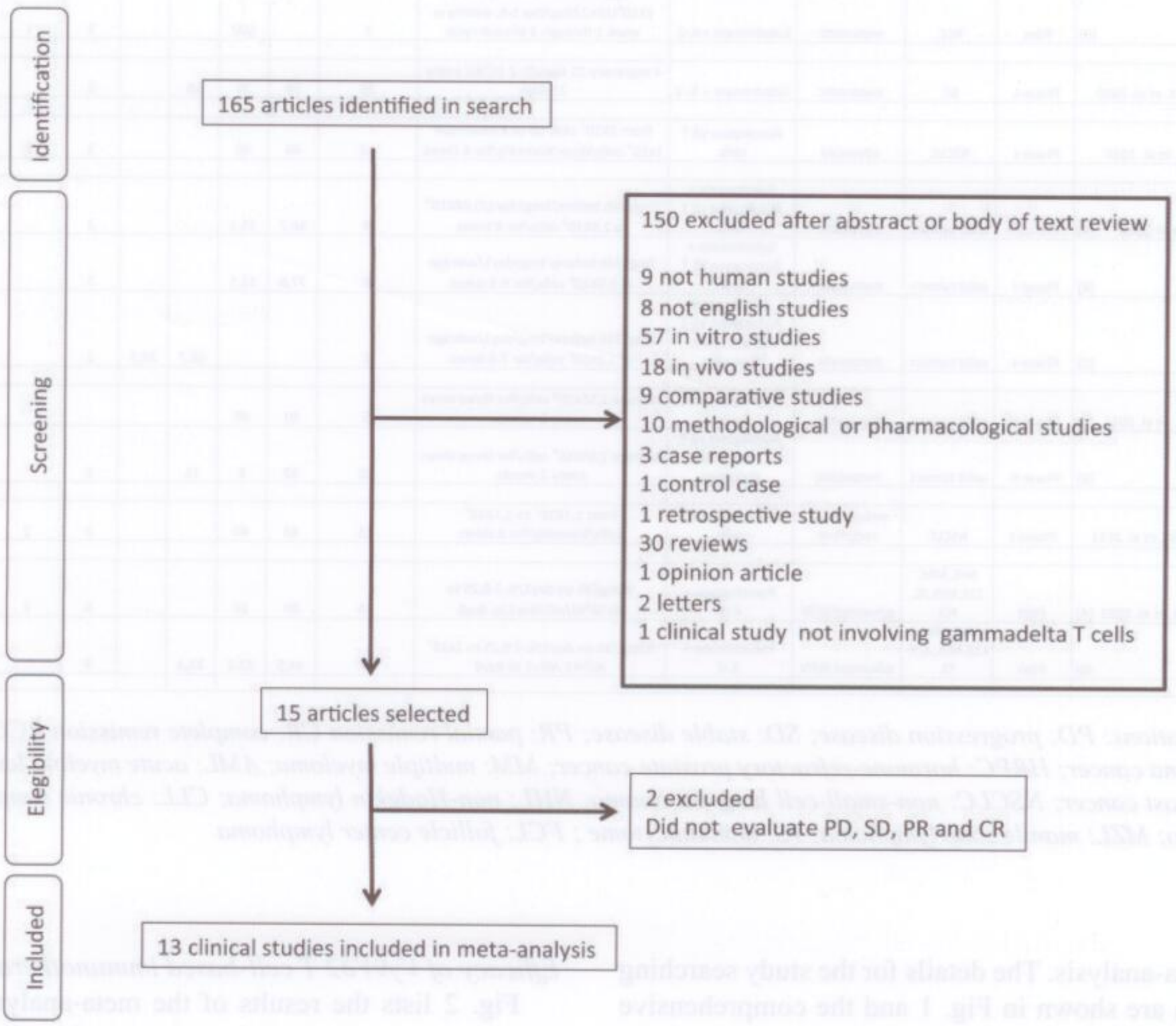

Fig. 1. *Flowchart showing record identification, record screening, full text article eligibility and study inclusion process.*

Table I. *Overview of 13 studies included in the meta-analysis (N=204).*

Source (Reference)	Trial Phase	Diagnosis	Stage of patients	Treatment design	Dosage	No. of patients	% of events/No. of patients				No. of patients with AEs		
							PD	SD	PR	CR	Mild	Moderate	Severe
Bennouna J. et al. 2008	Phase-I	RCC	metastatic	Autologous γδ T cells (Innacell gammadelta TM) + IL-2	1,4,8 and 12X10^9 cells/every 21 days/2X10^6 IU/m2/2 times for day /day 21 to day 28- day 42 to day 48	10	40	60			6	3	1
Bennouna J. et al. 2010	Phase-I	solid tumors	advanced or metastatic	BrHPP (IPH1101)+IL-2	200mg/m2 for1 time in 21 days/ 600, 1200, 1800 and 2400 mg/m2/every 21 days/day 1-7/IL-2 1X10^6 IU/m2/day	28		42,8			26	2	
Dieli F. et al. 2007 (A)	Phase-I	HRPC	metastatic	Zoledronate + IL-2	4 mg/every 21 days/IL-2 0,6X10^{6}IU/ every 21 days/for 1 year	9	33,3	44,5	22,2		6		
(B)	Phase-I	HRPC	metastatic	Zoledronate	4 mg/every 21 days/for 1 year	9	78	11	11		2		
Kobayashi H. et al. 2007	Pilot	RCC	metastatic or recurrent	Autologous γδ T cells + IL-2	0,7 x10^6 cells/day1/ IL-2/week	7			42,8		7		
Kobayashi H. et al. 2011	Phase I/II	RCC	metastatic	Zoledronate+ autologous γδ T cells+ IL-2	4 mg/cells/IL-2 1,4X10^6 UI/day/day1-5/6 times once every 4 weeks	11	45,4	45,4		9,2	10		
Kunzmann V. et al. 2012	Phase I/II	RCC, MM or AML	advanced or metastatic	Zoledronate +IL-2	4mg/day1 every 28 days/ IL-2 1X10^{6}U/m2/day/day 1-6/ up to a total of 6 cycles	21	57,1	28,6	9,5		21	2	
Lang J.M. et al. 2011 (A)	Pilot	RCC	metastatic	Zoledronate + IL-2	4mg/day1 every 28 days/ IL-2 7X10^{6}U/m2/day/day 1-5, weekly in week 1 through 3 of each cycle	9	11,1	44,4			7	3	3
(B)	Pilot	RCC	metastatic	Zoledronate + IL-2	4mg/day1 every 21 days/ IL-2 1X10^{6}U/m2/day/day 1-5, weekly in week 1 through 3 of each cycle	3		100			3	1	
Meraviglia S. et al. 2010	Phase-I	BC	metastatic	Zoledronate + IL-2	4 mg/every 21 days/IL-2 10^{6}IU/ every 21 days	10	70	20	10		6		
Nakajima J. et al. 2010	Phase-I	NSCLC	advanced	Autologous γδ T cells	from 1X10^7 cells up to a maximum 1x10^9 cells/dose/biweekly/for 6 times	10	40	40			3	2	
Nicol A.J. et al. 2011 (A)	Phase-I	solid tumors	metastatic	Zoledronate + Autologous γδ T cells	1mg/24h before/1mg/day1/0,04X10^9 to 2,8X10^9 cells/for 8 times	6	66,7	33,3			2		
(B)	Phase-I	solid tumors	metastatic	Zoledronate + Autologous γδ T cells	1mg/24h before/1mg/day1/average of 0,9X10^9 cells/for 6-8 times	9	77,8	11,1			3		
(C)	Phase-I	solid tumors	metastatic	Zoledronate+ Autologous γδ T cells + other therapies	1mg/24h before/1mg/day1/average of 1,2X10^9 cells/for 7-8 times	3			66,7	33,3	2		
Noguchi A. et al. 2011 (A)	Phase-II	solid tumors	metastatic	Autologous γδ T cells	average 2,55x10^9 cells/for three times every 2-weeks	5	40	40					
(B)	Phase-II	solid tumors	metastatic	Autologous γδ T cells + other therapies	average 2,55x10^9 cells/for three times every 2-weeks	20	30	5	15		2		
Sakamoto M. et al. 2011	Phase-I	NSCLC	metastatic or recurrent	Autologous γδ T cells	from 1,1X10^7 to 1,1X10^9 cells/biweekly/for 6 times	15	40	40			2	3	
Wilhelm M. et al. 2003 (A)	Pilot	NHL,MM, CLL,MZL,IC, FCL	advanced III/IV	Pamidronate + IL-2	90mg/3h on day1/IL-2 0,25 to 3x10^{6}IU/m2/day3 to day8	10	80	10			8	2	
(B)	Pilot	NHL,MM, CLL,MZL,IC,FCL	advanced III/IV	Pamidronate + IL-2	90mg/3h on day1/IL-2 0,25 to 2x10^6 IU/m2 /day1 to day6	9	44,5	22,2	33,3		8		

Abbreviations: PD: progression disease; SD: stable disease; PR: partial remission CR: complete remission RCC: renal carcinoma cancer; HRPC: hormone-refractory prostate cancer; MM: multiple myeloma; AML: acute myeloid leukemia; BC: breast cancer; NSCLC: non-small-cell lung carcinoma; NHL: non-Hodgkin lymphoma; CLL: chronic lymphocytic leukemia; MZL: mantle zone lymphoma; IC: immunocytome ; FCL: follicle center lymphoma.

for meta-analysis. The details for the study searching process are shown in Fig. 1 and the comprehensive characteristics of the 13 clinical studies included are shown in Table I.

Efficacy of Vγ9Vδ2 T cell-based immunotherapy

Fig. 2 lists the results of the meta-analysis and heterogeneity test: it shows that the objective anti-tumor response conferred by Vγ9Vδ2 T cell-based

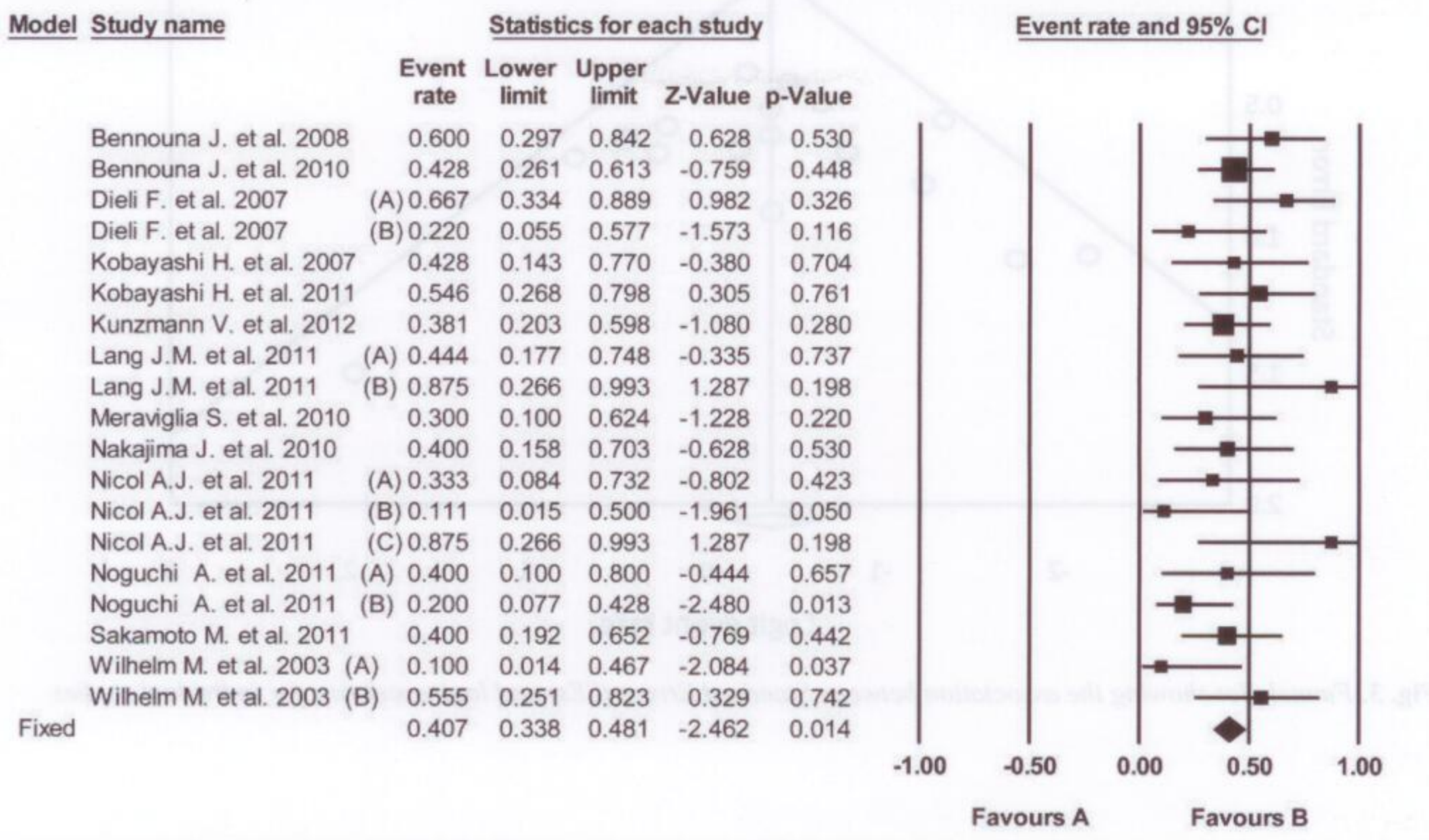

Fig. 2. *Forest plot of comparison of Vγ9Vδ2 T cell-based immunotherapy and clinical response is displayed. The size of the squares is proportional to the sample size. Horizontal lines denote 95% CI for single studies, the diamond the 95% CI for the overall Mantel-Haenszel estimate (fixed effect).*

immunotherapy in the overall 13 studies and groups reaches a significant difference. There was no evidence of heterogeneity among the overall studies (I^2 = 14.12%), suggesting that fixed effect model is appropriate. The estimated event rate was 0.407 (*P*-value = 0.014) for patients with SD or PR or CR.

To further strengthen the confidence for the results, we conducted a sensitivity analysis. First we computed the Begg's Funnel plot and Egger's test to access the publication bias of the studies. The shape of the Funnel plots was symmetrical, suggesting there is no evidence of publication bias among the studies (Fig. 3). The Egger's regression intercept was 0.24 (*P*-value>0.10) demonstrating no evidence for publication bias. Therefore, this sensitivity analysis confirmed the stability of the association between Vγ9Vδ2 T cell-based immunotherapy and the lack of disease progression.

We then measured the relative influence of each study on the pooled estimate by excluding a single study from analysis (data not shown). Together with the above reported results, this meta-analysis showed significant association between Vγ9Vδ2 T cell-based immunotherapy and the progression free survival.

Two different immunotherapy protocols have been used in the 13 selected studies (15-27): Vγ9Vδ2 T cells activated *in vivo* with PAgs/IL-2 or N-BPs/IL-2 and of the adoptive transfer of *ex vivo* preactivated autologous Vγ9Vδ2 T lymphocytes. We then compared patients treated with P-Ags or N-BPs plus IL-2 *in vivo* (16-22), with patients treated with adaptive transfer of *ex vivo*-expanded Vγ9Vδ2 T cells (22-26) and patients who received

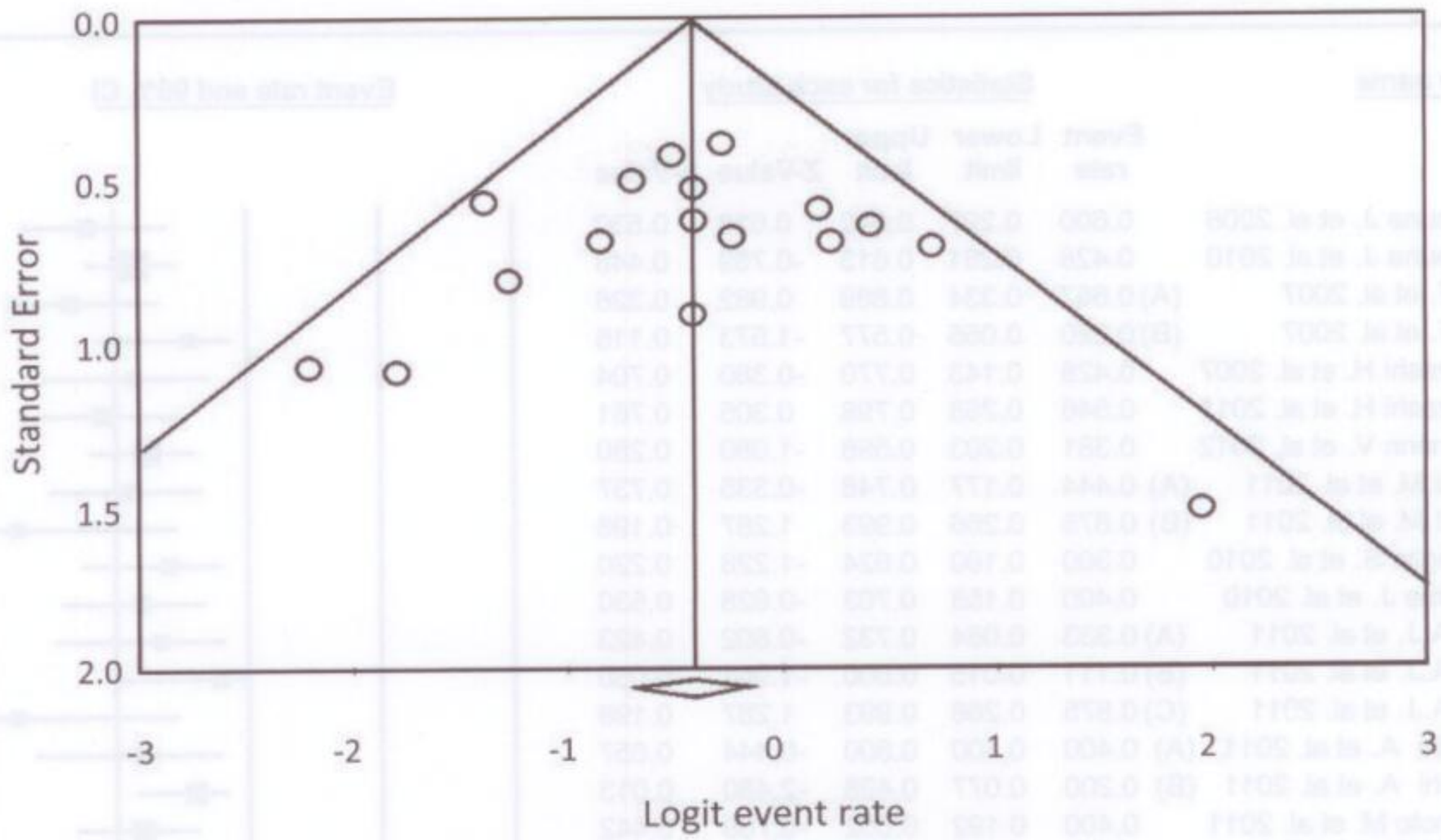

Fig. 3. *Funnel plot showing the association between Standard Errors (SEs) and logit event rate for individual studies.*

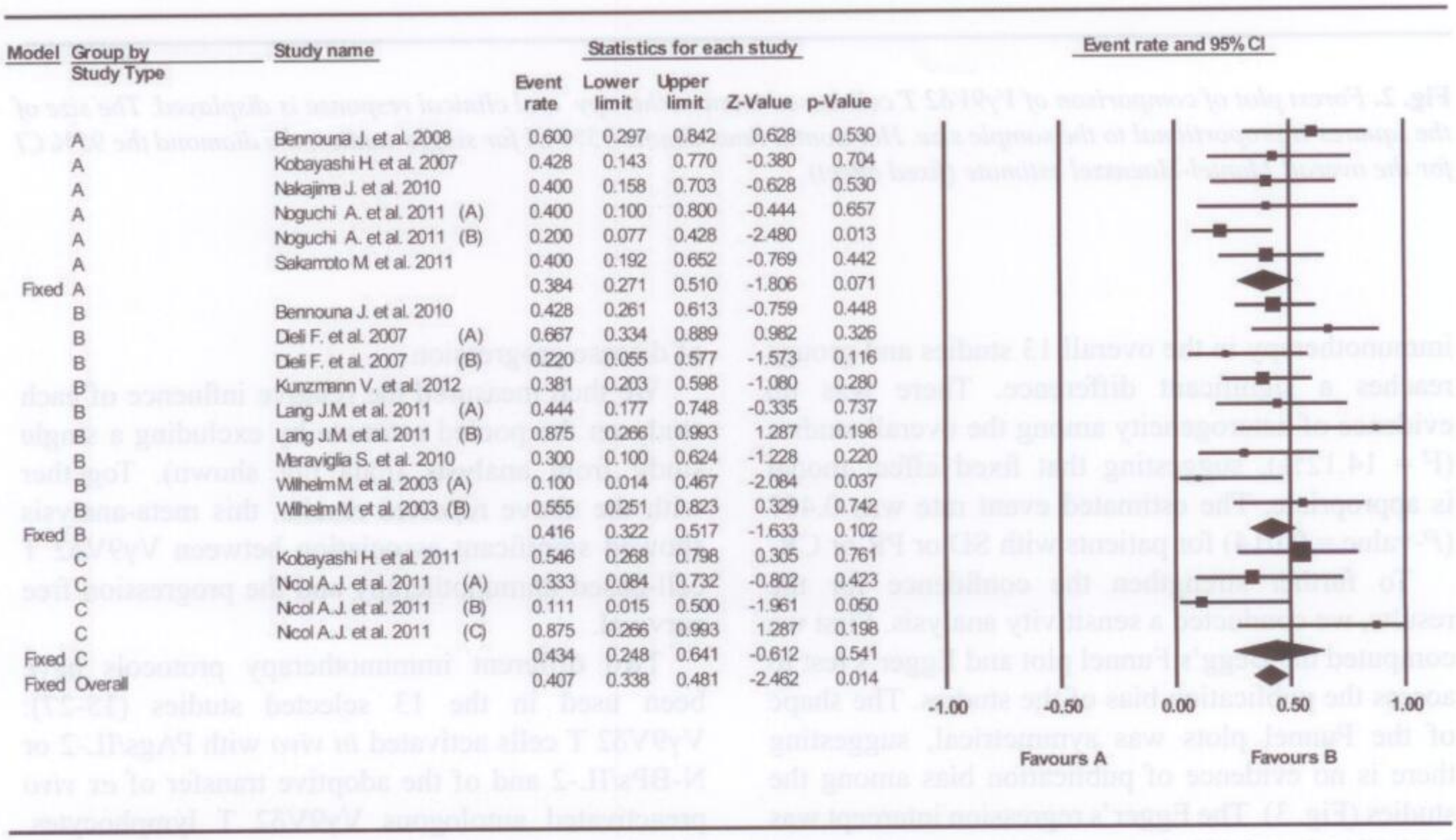

Fig. 4. *Forest plot of comparison of three different Vγ9Vδ2 T cell-based protocols and clinical response is displayed. The size of the squares is proportional to the sample size. Horizontal lines denote 95% CI for single studies, the diamond the 95% CI for the overall Mantel-Haenszel estimate (fixed effect).*

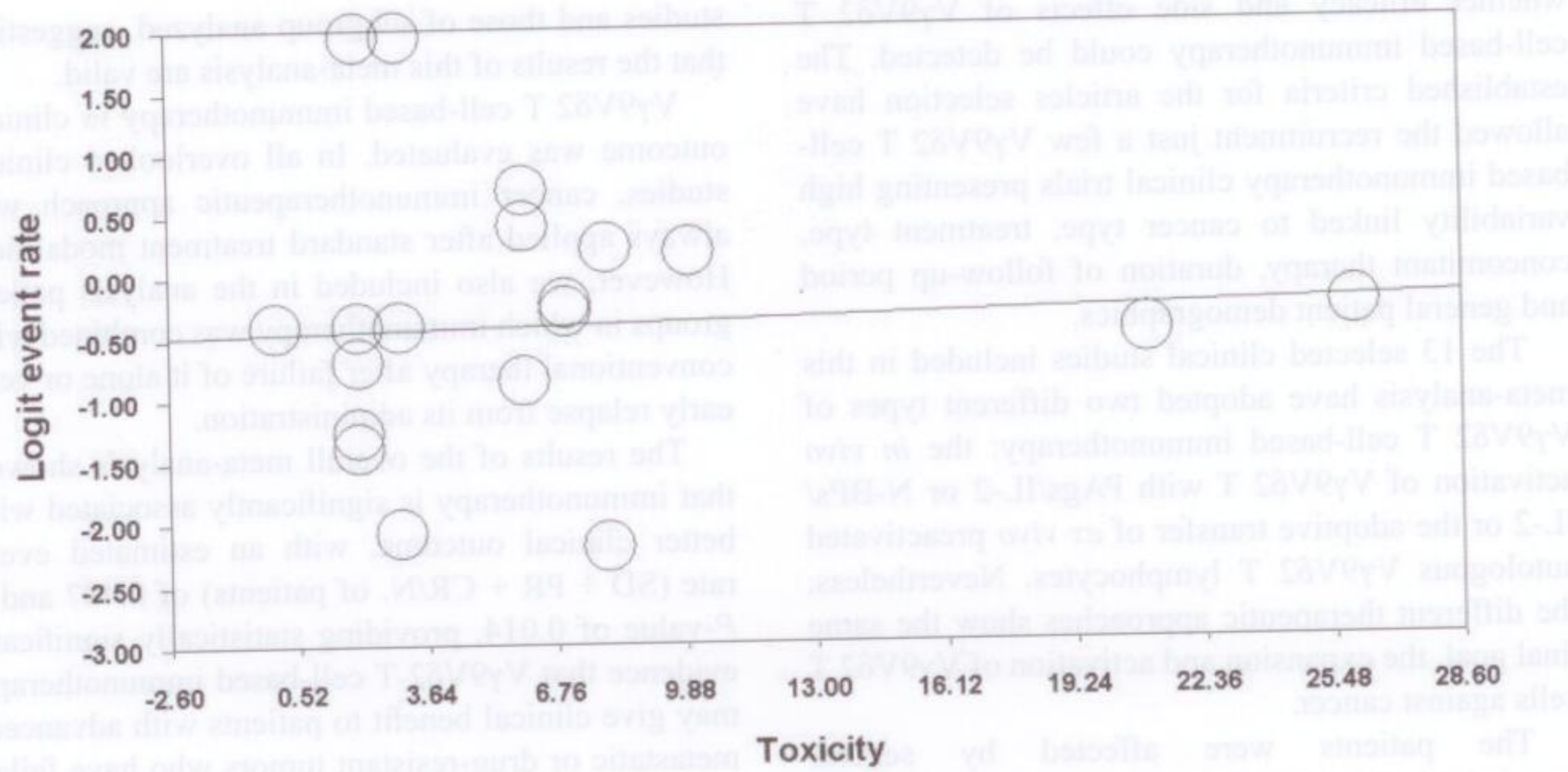

Fig. 5. *Forest plot of comparison of Vγ9Vδ2 T cell-based immunotherapy and adverse effects is displayed. The size of the squares is proportional to the sample size. Horizontal lines denote 95% CI for single studies, the diamond the 95% CI for the overall Mantel-Haenszel estimate (fixed effect)*

both treatments (15, 27). As shown in Fig. 4, there were not significant differences between the three tested groups (*P*-value > 0.05).

Vγ9Vδ2 T cell treatment-related toxicity

Safety analysis was based on the AEs detected by clinical and laboratory examination in the 13 trials (15-27). Only two studies have reported severe side effects: Bennouna et al. (23) reported one instance of disseminated intravascular coagulation, while Lang et al. (20) reported 3 instances of severe effects, elevation in creatinine levels, hyperglycemia and myocardial infarction, but this last was believed unrelated to study medication.

Among the reviewed 13 studies, most AEs were mild or moderate, and included typically flu-like syndrome, injection-site reaction, gastrointestinal disorders (abdominal pain, diarrhea) and hypotension.

To investigate the potential determinants of these AEs we then run a simple meta- regression of the logit event rate on the toxicity. Results (Fig. 5) show no association between Vγ9Vδ2 T cell treatment-related AEs and the immunotherapy efficacy (*P*-value > 0.05) indicating that Vγ9Vδ2 T-cell based immunotherapy does not correlate with AEs and, in turn, AEs do not influence or affect Vγ9Vδ2 T cell-based immunotherapy

DISCUSSION

A growing body of evidence indicates that gd T lymphocytes, and in particular their Vγ9Vδ2 subset, are important effector cells of the immune system that may play a role in the anti-tumor surveillance in peripheral tissues (1, 9, 10). Accordingly, several clinical trials involving patients with different advanced disease, resistant to conventional treatments, have been peformed to assay the safety and efficacy of Vγ9Vδ2 T cell-based immunotherapy (15-27). However, the heterogeneity of therapy algorithms, non standardized cellular products and the lack of established criteria for clinical responses have made it impossible to draw valid conclusions from single clinical trials. This has also been due to the design of clinical trials conducted so far, most being small phase I/II or pilot studies with conventional end points of feasibility. Therefore, the objective of this meta-analysis was to determine

whether efficacy and side effects of Vγ9Vδ2 T cell-based immunotherapy could be detected. The established criteria for the articles selection have allowed the recruitment just a few Vγ9Vδ2 T cell-based immunotherapy clinical trials presenting high variability linked to cancer type, treatment type, concomitant therapy, duration of follow-up period and general patient demographics.

The 13 selected clinical studies included in this meta-analysis have adopted two different types of Vγ9Vδ2 T cell-based immunotherapy: the *in vivo* activation of Vγ9Vδ2 T with PAgs/IL-2 or N-BPs/IL-2 or the adoptive transfer of *ex vivo* preactivated autologous Vγ9Vδ2 T lymphocytes. Nevertheless, the different therapeutic approaches show the same final goal, the expansion and activation of Vγ9Vδ2 T cells against cancer.

The patients were affected by several hematological and solid tumors (e.g. multiple myeloma, acute myeloid leukemia, non-Hodgkin lymphoma, hormone resistant prostate cancer, renal cell carcinoma, breast adenocarcinoma and others) in advanced or metastatic stage (15-27), with wide range of age and different demographical origin.

The variability of the different immunotherapy studies with respect to treatment modality (adoptive cell transfer *vs* PAgs or N-BPs and IL-2), number of treatments (single, repeated), cancer type (solid, haematological), concomitant therapy, general patient demographics and length of follow-up period, and the limitations of the study, should not technically allow for a meta-analysis to assess treatment outcomes across such diverse trials. However, this is a general problem that applies to all rare diseases and small-scale trials where it is impossible to reach sufficient statistical power.

Nonetheless, we pooled in this meta-analysis studies including different types cancers, our results showed no evidence of overall heterogeneity. Moreover, no significant publication bias existed. To avoid bias in the identification and selection of studies, as many non-randomized and randomized controlled trials as possible were included to improve the statistical reliability. The literature search strategy was designed to ensure that all important published trials were supervised. Finally, estimation of event rate demonstrated that no statistical inconsistency existed between the results from each of the original studies and those of subgroup analyzed, suggesting that the results of this meta-analysis are valid.

Vγ9Vδ2 T cell-based immunotherapy *vs* clinical outcome was evaluated. In all overlooked clinical studies, cancer immunotherapeutic approach was always applied after standard treatment modalities. However, we also included in the analysis patient groups in which immunotherapy was combined with conventional therapy after failure of it alone or very early relapse from its administration.

The results of the overall meta-analysis showed that immunotherapy is significantly associated with better clinical outcome, with an estimated event rate (SD + PR + CR/N. of patients) of 0.407 and a *P*-value of 0.014, providing statistically significant evidence that Vγ9Vδ2 T cell-based immunotherapy may give clinical benefit to patients with advanced/metastatic or drug-resistant tumors who have failed conventional therapies.

In the selected trials two kinds of immunotherapy were administrated: *in vivo* activation of Vγ9Vδ2 T with PAgs/IL-2 or N-BPs/IL-2 or the adoptive transfer of *ex vivo* preactivated autologous Vγ9Vδ2 T lymphocytes. As the PR rate was higher in the subgroup of patients receiving *in vivo* PAgs or N-BPs and IL-2 (Table I), we also evaluated if differences existed between patients undergoing the two kinds of immunotherapeutic regimens; comparison also included 2 clinical studies (15, 27) in which patients received both treatments. As shown in Fig. 4, our meta-analysis did not highlight any statistically significant difference between the three analyzed groups.

Vg9Vd2 T cell-based immunotherapy induced treatment-related AEs. Occurrence of a total of 4 severe side effects were reported by two studies (20, 23) (but one of such AEs was believed unrelated to study medication (20), while the great majority of AEs were mild or moderate: approximately 40% of patients treated with i.v. N-BPs manifested an acute-phase response after the first administration of the drug (29). This is characterized by a flu-like syndrome, with fever, fatigue, malaise and myalgia, arthralgia, and bone pain (29). It is benign and self-limited and is consequent to the immune response induced by the N-BP, caused by the release of cytokines by Vγ9Vδ2 T cells and macrophages (29). It has been speculated that the occurrence of the flu-like syndrome, reflecting overall Vγ9Vδ2 T

cell responsiveness, should be predictive of further clinical response *in vivo*: however, results of this meta-analysis demonstrate that AEs do not influence the efficacy of Vγ9Vδ2 T cell-based immunotherapy and *v.v.*

Vg9Vd2 T cell-based clinical trials have defined conditions for the safe use of P-Ags and N-BPs for the activation of these cells in patients *in vivo*; similarly, immunotherapy based on the adoptive transfer of *ex vivo* preactivated, autologous Vγ9Vδ2 T lymphocytes is now feasible and safe but technically more demanding than the former (30). Clearly, advantages of the adoptive therapy are the ability to control cell expansion and to modify the growing cells throughout the culture process, for example by supplementation of the cultures with selected cytokines. Moreover, this method makes it possible to additionally treat patients who cannot or have failed to respond to injection of PAgs or N-BPs and IL-2. Furthermore, the lack of MHC restriction theoretically opens the possibility of expanding *ex vivo* allogeneic Vγ9Vδ2 T lymphocytes, to produce batches of several billion Vγ9Vδ2 T lymphocytes to re-inject into patients Protocols aimed to combine chemotherapy and therapeutic monoclonal antibodies are now rapidly progressing through phase I-III trials, and some clinical successes seem to emerge (30). Therefore, novel regimens that combine such drugs with Vγ9Vδ2 T cell-based strategies, should be taken into consideration.

In conclusion, gd T cell-based immunotherapy shows a statistically significant advantage for SD, PR and CR in patients with hematological and solid malignancies and it also produces a low-grade toxicity. The results of this meta-analysis, despite its limitation, confirm that alone or in combination regimens gd T cell-based immunotherapy can be used in all patients affected by metastatic resistant/ refractory cancer. Further investigation in phase III randomized trials are required to finally demonstrate the clinical benefit of Vγ9Vδ2 T cell -based immunotherapy.

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