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Brief Report

Meta-Analysis Reveals Significant Sex Differences in Chronic Lymphocytic Leukemia Progression in the *Eμ-TCL1* Transgenic Mouse Model

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Abstract: The *Eμ-TCL1* transgenic mouse model represents the most widely and extensively used animal model for chronic lymphocytic leukemia (CLL). In this report, we performed a meta-analysis of leukemia progression in over 300 individual *Eμ-TCL1* transgenic mice and discovered a significantly accelerated disease progression in females compared to males. This difference is also reflected in an aggressive CLL mouse model with additional deletion of *Tp53* besides the *TCL1* transgene. Moreover, after serial adoptive transplantation of murine CLL cells, female recipients also succumbed to CLL earlier than male recipients. This sex-related disparity in the murine models is markedly contradictory to the human CLL condition. Thus, due to our observation we urge both careful consideration in the experimental design and accurate description of the *Eμ-TCL1* transgenic cohorts in future studies.

Keywords: chronic lymphocytic leukemia; sex difference; *TCL1*; transgenic mouse model; adoptive transplantation

1. Introduction

Increasing understanding of the biology and pathogenesis of chronic lymphocytic leukemia (CLL) has led to many breakthroughs in the treatment of this disease [1,2], which has been acquired owing considerably to the use of animal models. To date, the *Eμ-TCL1* transgenic mouse model is the most widely used CLL model, indicated by over 500 citations of the original paper [3]. The ectopic expression of the human T cell leukemia 1 (*TCL1*) oncogene under the control of the V_H-promoter and Ig_H-Eμ-enhancer in transgenic mice enables the development of a highly similar CLL-like disease with 100% penetrance. The close resemblance of the human CLL disease and high penetrance in the *Eμ-TCL1* transgenic mice renders this model a popular tool to study pathogenic interactions leading to CLL. The *Eμ-TCL1* transgenic mice have been extensively used in the field of CLL research, in which these mice either were intercrossed with a vast variety of mice bearing other mutations or served as

a pre-clinical model for different treatment options [4–7]. Given their intensive usage, a thorough understanding of CLL pathogenesis in *Eμ-TCL1* transgenic mice is critical for the precise interpretation of the acquired results.

The prevalent influences of sex on the disease phenotypes of many mouse lines have been evaluated [8], including several mouse models for cancers [9–11]. Although the incidence and clinical course of CLL is strongly different between men and women [1], the sex-related characteristics of the CLL mouse model remain unexplored. Here, we report a profound difference in the leukemic progression and overall survival between male and female *Eμ-TCL1* transgenic mice, which is markedly contradictory to the human CLL condition. Our finding based on the analysis of over 300 mice argues for unbiased experiment design and more accurate description of the *Eμ-TCL1* transgenic mouse cohorts in future studies.

2. Materials and Methods

2.1. Mouse Cohorts and Housing Facilities

All mouse experiments were approved by the state authorities of North Rhine–Westphalia, Germany (LANUV) #K17,12/04; #9.93.2.10.31.07.097; #9.93.2.10.31.07.098; #8.87-50.10.37.09.241; #84-02.04.2014.A146; #84-02.04.2016.A058. All analyzed mice were hemizygote for the *TCL1* transgene (*Eμ-TCL1*$^{tg/wt}$), had either a hybrid C3H/HeJ × C57BL/6 (B6C3) or a C57BL/6 (B6) genetic background, and were housed in groups of up to five animals per cage in individually ventilated cages (IVC) in three different animal facilities of the University Hospital of Cologne (Table 1). Mice housed in the Institute of Experimental Medicine (EM) and the Institute of Pathology (PA) were specific pathogen-free (SPF), mice housed in the facility of the CECAD Research Center were specific and opportunistic pathogen-free (SOPF). Mice from four cohorts harbored additional, unaffecting mutations besides the *TCL1* transgene (Table 1).

Table 1. Characteristics of the analyzed *Eμ-TCL1*$^{tg/wt}$ mice.

No.	Ref.	Genetic Background	Additional, Unaffecting Transgene	Housing Facility	Number of Mice			Median Survival (Days)		
					Total	Male	Female	Male	Female	P (Log-Rank)
1	[12]	B6C3	-	EM	41	17	24	378	339.5	0.0080
2	[13]	B6C3	-	EM	36	15	21	424	369	0.0808
3	[14]	B6C3	-	EM	38	16	22	454.5	376.5	0.0025
4	[15]	B6 (J/N-mix)	Hemizygote CD19-Cre transgene without any loxP-flanked allele	PA	13	8	5	369	320	0.9621
5	-	B6 (J/N-mix)	LoxP-flanked mutant alleles without any Cre transgene	EM	29	17	12	402	358	0.2971
6	-	B6C3	-	EM	43	21	22	425	404.5	0.2700
7	-	B6C3	LoxP-flanked mutant alleles without any Cre transgene	EM	6	3	3	430	434	0.6537
8	-	B6C3	LoxP-flanked mutant alleles without any Cre transgene	EM	22	10	12	429.5	400.5	0.5522
9	-	B6C3	-	EM	47	28	19	357	345	0.2092
10	-	B6 (J)	-	CE	21	10	11	443	389	0.0466
11	-	B6 (J/N-mix)	-	PA	22	15	7	360	308	0.0079

2.2. Mouse Blood Analyses

Procedures for differential blood counts and determination of CLL burden were previously described [12–15]. Leukocyte count (LC) was measured with a SYSMEX XE-5000 system (Sysmex,

Kobe, Japan). CLL cells defined as $CD19^+$ $CD5^+$ or IgM^+ $CD5^+$ were identified by flow cytometry with either a BD FACSCanto (BD, Franklin Lakes, New Jersey), or a Gallios (Beckman Coulter, Brea, CA, USA), or a MACSQuant *VYB* or a MACSQuant *X* (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were analyzed using Kaluza Flow Analysis Software (Beckman Coulter) or FlowJo™ Analysis Software (BD).

2.3. Survival Determination

Mice reaching endpoint defined by the animal welfare law were euthanized by cervical dislocation. All death events unrelated to leukemia were excluded from this study. Survival of *Eμ-TCL1* transgenic mice was recorded from birth until death or euthanasia. Survival of transplanted recipients was recorded from the day of CLL injection until death or euthanasia.

2.4. Syngeneic Adoptive Transplantation

Freshly homogenized and filtered splenocytes from moribund *Eμ-TCL1* transgenic mice of pure C57BL/6 (J) genetic background were layered with Pancoll separating solution (PAN Biotech, Aidenbach, Germany), followed by centrifugation and separation of interphase-concentrated mononuclear cells. After several washing steps, 10^7 cells were injected intraperitoneally into sex-matched C57BL/6 (J) mice between eight and 14 weeks of age, generating Passage 1 (P1) recipients. Similar procedures were applied in Passage 2 (P2) transplantation, in which splenocytes of moribund P1 recipients were injected into P2 recipients.

2.5. Statistical Analysis

All statistical differences of blood data were calculated with the Mann–Whitney test, comparisons of the survival curves were calculated with the Mantel–Cox logrank test using Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Results

We assessed CLL progression in male and female *Eμ-TCL1* transgenic mice in 11 independent studies ([12–15] and unpublished data) regarding the percentage of CD5 positive B-CLL cells in the murine peripheral blood (% CLL) at six and 12 months, and the overall survival (OS). Our meta-analysis of all *Eμ-TCL1*$^{tg/wt}$ mice revealed a significantly slower CLL progression in males compared to their female counterparts. At six months—the estimated time of disease establishment—% CLL was significantly lower by 7.876% in males versus females (Males: n = 123; Mean ± SEM: 17.55% ± 1.435%. Females: n = 121; Mean ± SEM: 25.24% ± 1.953%) (Figure 1A). At this time point, the mean leukocyte count in blood (LC) was slightly lower in males compared to females by 3,136 cells/μL (Males: n = 174; Mean ± EM: 18,420 ± 821.3. Females: n = 116; Mean ± SEM: 21,556 ± 3112) (Figure 1B). The difference in CLL burden became more compelling at the fully developed disease state of 12 months, the sex difference in % CLL increased to 15.37% (Males: n = 95; Mean ± SEM: 43.87% ± 2.758%. Females: n = 60; Mean ± SEM: 59.24% ± 3.701%) (Figure 1C), the LC difference increased to 30,861 cells/μL (Males: n = 97; Mean ± SEM: 45,581 ± 8,089. Females: n = 61; Mean ± SEM: 76,442 ± 16,062) (Figure 1D).

The substantial reduction in the number of females at 12 months as a consequence of their shorter OS also indicates the more progressive leukemic courses in females. In 10 mouse cohorts that were characterized independently by different investigators, a longer OS of the males could be congruently observed, independently of the mouse genetic background and housing facility (Table 1). Altogether, males (n = 160) had a median OS of 397 days, whereas the median OS of females (n = 158) was only 360 days, implying a significantly longer OS of 37 days in males than females (Figure 2A). Recently, the *Eμ-TCL1* transgenic mice have been crossed with other mouse lines lacking tumor-suppressor genes [15] or harboring an additional oncogene [16], leading to accelerated leukemia development and enhanced disease aggressiveness in these mice. To investigate whether these additional transgenic alleles could compromise the male–female bias in the *Eμ-TCL1* transgenic mice, we analyzed the OS of

Eμ-TCL1$^{tg/wt}$; *CD19Cre*$^{Cre/wt}$; *Trp53*$^{fl/fl}$ (TCP) mice—a mouse model with features of high-risk human CLL. Due to B cell-specific deletion of *Tp53*, the TCP mice showed earlier disease onset, accelerated disease progression with occasional Richter transformation, and a significantly shorter lifespan than *Eμ-TCL1*$^{tg/wt}$ mice [15]. Despite a smaller cohort of TCP mice in our analysis, a clear trend in sex difference could also be observed. Independent of their genetic background and housing condition (Table 2), TCP males had a survival benefit of 32 days compared to TCP female littermates (Males: n = 29, median OS: 252 days, Females: n = 27, median OS: 220 days) (Figure 2B). This result suggests that the sex difference in leukemia development might be passed on to other mouse models with additional genetic lesions that were crossbred with the *Eμ-TCL1* transgenic mice. Of note, the shorter survival in females appeared to be a sole effect of *TCL1*-induced CLL in mice, because wildtype (WT) females lived longer than WT males in the same animal husbandry (Figure 2C). These WT mice are littermates of the *Eμ-TCL1*$^{tg/wt}$ mice in cohort #3 and #5 (Table 1).

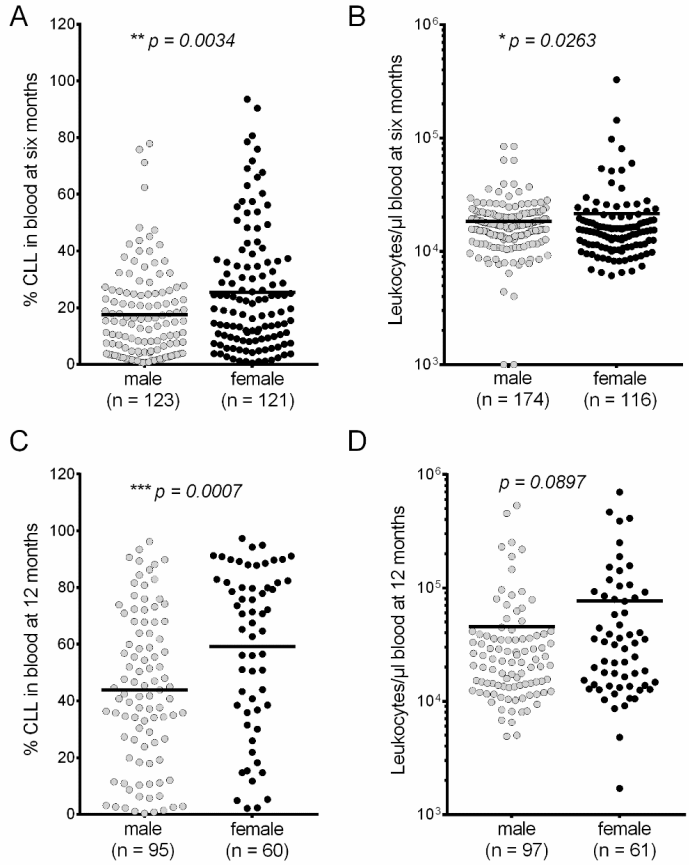

Figure 1. Significant sex difference in chronic lymphocytic leukemia (CLL) progression in the *Eμ-TCL1*$^{tg/wt}$ mice. (**A**) Significantly lower CLL load in the peripheral blood of males compared to females at six months. Blood samples from tail vein were assessed by flow cytometry to determine the CD5-positive CLL percentage in lymphocytes. Males: n = 123; Mean ± SEM: 17.55% ± 1.435%. Females: n = 121; Mean ± SEM: 25.43% ± 1.953%. Mann–Whitney test: ** p = 0.0034. (**B**) Lower leukocyte count in the peripheral blood (cells per µL) in male than female *Eμ-TCL1*$^{tg/wt}$ mice at six months. Males: n = 174; Mean ± SEM: 18,420 ± 821.3; Females: n = 116; Mean ± SEM: 21,556 ± 3112; Mann–Whitney test: * p = 0.0263. (**C**) Significantly lower CLL load in the peripheral blood of males compared to females at 12 months. Males: n = 95; Mean ± SEM: 43.87% ± 2.758%. Females: n = 60; Mean ± SEM: 59.24% ± 3.701%. Mann–Whitney test: *** p = 0.0007. (**D**) Lower leukocyte count in the peripheral blood (cells per µL) in male than female *Eμ-TCL1*$^{tg/wt}$ mice at 12 months. Males: n = 97; Mean ± SEM: 45,581 ± 8,089; Females: n = 61; Mean ± SEM: 76,442 ± 16,062; Mann–Whitney test: p = 0.0897.

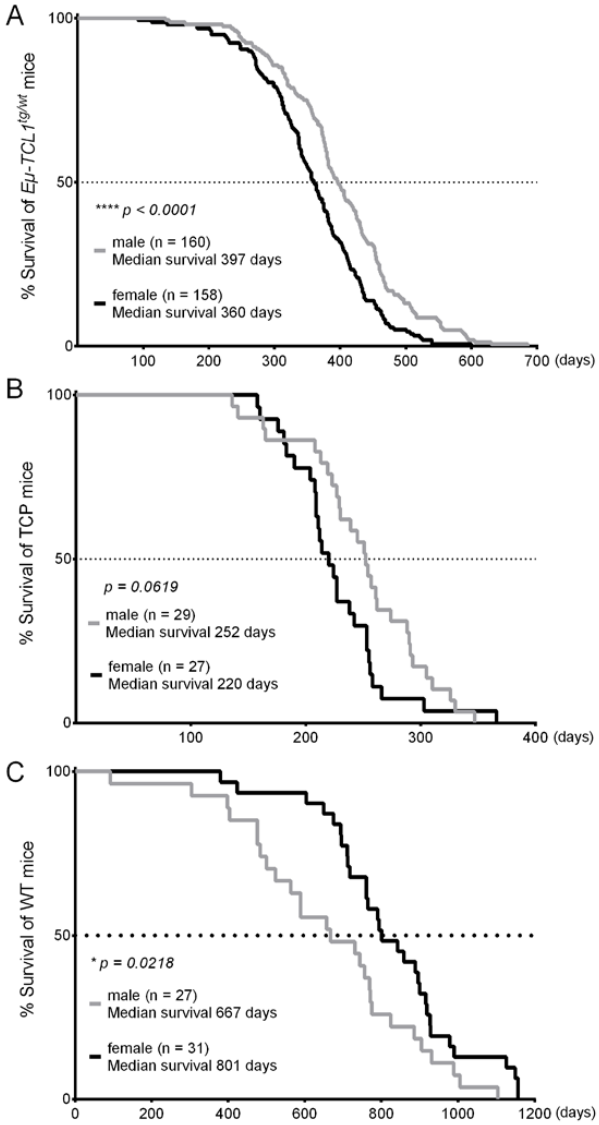

Figure 2. Significant sex difference in the overall survival of *Eμ-TCL1*$^{tg/wt}$ and *Eμ-TCL1*$^{tg/wt}$; *CD19Cre*$^{Cre/wt}$; *Trp53*$^{fl/fl}$ (TCP) mice. (**A**) Kaplan–Meier curves representing the overall survival of 160 male and 158 female *Eμ-TCL1*$^{tg/wt}$ mice showed significantly longer survival of males than females. Median survival: *Eμ-TCL1*$^{tg/wt}$ males = 397 days; *Eμ-TCL1*$^{tg/wt}$ females = 360 days. Mantel–Cox logrank test: **** $p < 0.0001$. (**B**) Kaplan–Meier curves representing the overall survival of 29 male and 27 female TCP mice demonstrating the longer survival of males than females. Median survival: TCP males = 252 days; TCP females = 220 days; Mantel–Cox logrank test: $p = 0.0619$. (**C**) Kaplan–Meier curves representing the overall survival of 27 male and 31 female wild type (WT) mice showed significantly longer survival of females compared to males, in contrast to the sex difference observed in the *Eμ-TCL1* transgenic mice. Median survival: WT males = 667 days, WT females = 801 days; Mantel–Cox logrank test: * $p = 0.0218$.

Table 2. Characteristics of the analyzed *Eμ-TCL1*$^{tg/wt}$; *CD19Cre*$^{Cre/wt}$; *Trp53*$^{fl/fl}$ (TCP) mice.

No.	Ref.	Genetic Background	Housing Facility	Number of Mice			Median Survival (Days)		
				Total	Male	Female	Male	Female	P (Log-Rank)
1	[15]	B6 (J/N-mix)	PA	21	13	8	252	205.98	0.0223
2	-	B6 (J)	CE	35	16	19	256.5	227	0.2633

Recently, the adoptive transplantation of murine CLL cells in immunocompetent mice, which significantly accelerates the CLL course in recipients compared to the *Eμ-TCL1*transgenic mice, is increasingly used, particularly in studies involving the CLL tumor microenvironment [14,17–19]. To determine if the sex difference in *Eμ-TCL1*$^{tg/wt}$ mice can also be observed in the adoptive transfer setting, we performed sequential transplantation of *Eμ-TCL1*$^{tg/wt}$ leukemia cells in syngeneic, immunocompetent recipient mice. Using strictly syngeneic donor and recipient mice of the C57Bl/6 (J) genetic substrain, 10^7 CLL cells from each leukemic donor were injected peritoneally into sex-matched WT recipients. Here, Passage 1 (P1) male recipients lived 23 days longer than P1 females (Figure 3A), suggesting a tendency of sex disparity in the transplantation model. In a second transfer into Passage 2 (P2) recipients, a longer survival of 23 days could also be observed in P2 males compared to P2 females (Figure 3B), which represents a significant difference due to the shortened survival of P2 recipients compared to P1 recipients. In contrary to the sex-matched transplantation that always ensured engraftment and CLL progression in recipient mice, some sex-mismatched transplantation of leukemia cells failed to induce CLL, highlighting the importance of a sex-matched environment for CLL cells to engraft. In a few cases where CLL cells could be detected in sex-mismatched recipients, mismatched recipients had delayed disease onset and longer survival than sex-matched recipients receiving the same CLL cell clones. Of note, both male and female leukemia cells could grow in sex-mismatched recipients, which allows the sole effect of an immune response against the H-Y antigen to be excluded.

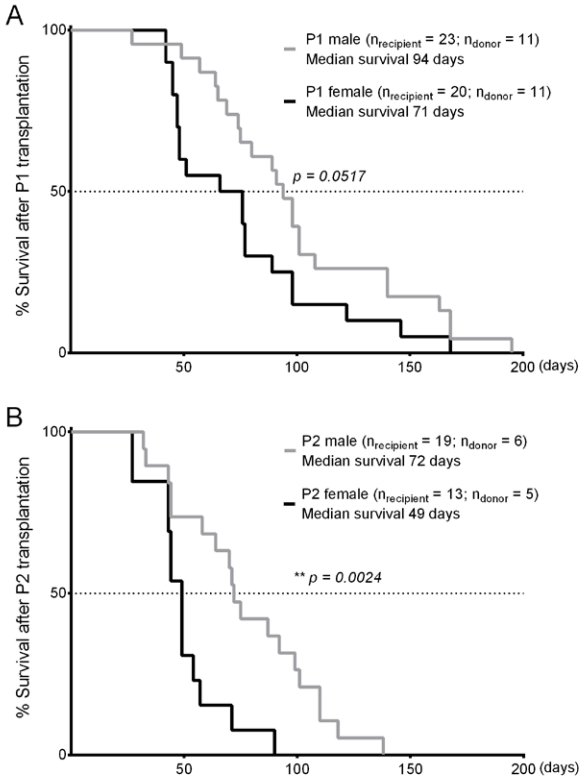

Figure 3. Significant sex difference in the survival of recipients after chronic lymphocytic leukemia (CLL) transplantation. (**A**) Kaplan–Meier curves of Passage 1 (P1) wild type (WT) recipient mice after syngeneic transplantation with murine CLL cells showing longer survival of males than females. Eleven male donor clones were transplanted in 23 male recipients; Eleven female clones were transplanted in 20 female recipients. Median survival: P1 males = 94 days, P1 females = 71 days; Mantel-Cox logrank test: $p = 0.0517$. (**B**) Kaplan–Meier curves of Passage 2 (P2) WT recipient mice after serial syngeneic transplantation with murine CLL cells showing longer survival of males than females. Six P1 male clones were transplanted in 19 male recipients; Five P1 female clones were transplanted in 13 female recipients. Median survival: P2 males = 72 days, P2 females = 49 days; Mantel–Cox logrank test: ** $p = 0.0024$.

4. Discussion

Our analyses of over 300 *Eμ-TCL1*$^{tg/wt}$ mice and 56 TCP mice, together with the serial adoptive transplantation experiment revealed a significantly accelerated CLL progression followed by shorter survival in females compared to males. Despite the variable factors that might interfere with data acquisition such as husbandry, handling, and mouse genetic substrain, this sex difference in CLL progression is highly consistent in our *Eμ-TCL1*$^{tg/wt}$ study cohorts. Over a decade of breeding the *Eμ-TCL1*$^{tg/wt}$ mice with WT mice, the hereditary pattern in our cohorts allow the X-linked inheritance of the *TCL1*-transgene to be excluded. Furthermore, the highly complex and heterogeneous genetic landscape of murine *TCL1* tumors was recently elucidated by whole exome sequencing, which did not disclose major abnormalities in the sex chromosomes [20]. Although a causal genetic variation in sex chromosomes cannot be entirely excluded at the moment, it seems more likely that our observed difference between male and female *Eμ-TCL1*$^{tg/wt}$ mice is an effect of autosomal variants or of sex-specific features such as hormones, metabolisms, or epigenetic alterations. These factors are not only distinctive between the sexes but also have critical influences on cancer susceptibility and tumor growth [21]. Interestingly, *TCL1* as the oncogenic driver of this CLL mouse model was shown to be regulated by estrogen [22,23], to be involved in critical cancer-related metabolic pathways including glycolysis [24,25], and to inhibit DNA methylations [26,27]. Moreover, the stark differences in the immune cell subsets [28] and immune responses [29] between male and female mice might also contribute to the unequal leukemic growth, particularly in a malignancy strongly dependent on the immune niche such as CLL [18,30]. Thus, further studies to compare the molecular signatures of leukemic and immune cells in males versus females, or analyses of CLL development in *Eμ-TCL1*$^{tg/wt}$ mice with hormone therapy might be helpful approaches to identify the determinant factors underlying this sex difference.

In particular, our observation revealed a discrepancy of sex influence on disease progression in the *Eμ-TCL1* transgenic mouse model compared to CLL patients. Men are not only twice as likely to develop CLL, but also have a worse prognosis than women [1]. Several studies have consistently reported that female CLL patients had more benign clinical courses including reduced incidence, superior 10-year survival, and better treatment response [31–33]. In CLL cases with unmutated *IGHV* genes that can be modelled in the *Eμ-TCL1* transgenic mice [20,34], female patients also showed significantly longer survival [32]. Although the explicit elements underlying the gender disparity in human CLL still remain largely unknown, the levels of circulating sex hormones or altered DNA methylation in sex-related gene promoters have been suggested to be relevant factors contributing to the better prognosis of women [35,36].

This sharp contradiction to the human condition in the *Eμ-TCL1* transgenic mice might represent a drawback in modelling CLL and requires further investigations. Meanwhile, this sex difference should be promptly addressed during experimental design, data interpretation, and publications. First and foremost, comparisons between a male-excessive and a female-excessive cohort must be avoided [37]. Moreover, the generation of additional CLL models should be facilitated [38,39], with consideration to any possible disparity in CLL development between the sexes.

5. Conclusions

The use of both sexes and of sex-matched animal cohorts has been implemented in the standard procedure for animal studies [40] and should be reported transparently and precisely in publications. However, the number of male and female mice was only specified in a very limited number of papers involving the *Eμ-TCL1* transgenic mice, most likely due to unawareness of the significant sex difference in this model. Based on the results of this study, together with the report on the importance of the genetic background of *Eμ-TCL1* transgenic mice in the adoptive transplantation setting [41], we urge for more transparent, accurate descriptions of animal models in future publications, including specification of the male-to-female ratio in each study cohort and the accurate genetic substrains of the mice.

Author Contributions: M.K. and P.-H.N. collected data, performed the meta-analyses and wrote the manuscript. M.K., S.R., J.S., N.R. (Natascha Rosen), O.F., G.K., L.T., R.B., N.R. (Nina Reinart) and P.-H.N. performed experiments. T.S.-N., H.C.R., M.H., and P.-H.N. initiated and supervised the individual studies. All authors have read and agreed to the published version of the manuscript.

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