Received Date : 24-Jul-2015
Revised Date : 06-Oct-2015
Accepted Date : 20-Oct-2015
Article type : Original

Prospective neonatal screening for severe T- and B- lymphocyte deficiencies in Seville

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pai.12501
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Funding resources:

This work was funded by

- Fundación Pública Andaluza para la Gestión de la Investigación en Salud de Sevilla, Hospital Universitario Virgen Del Rocío (FISEVI, “Scott Family Funding”)
- Fondo de Investigaciones Sanitarias (FIS), Instituto de Salud Carlos III (PI13/01104)
- Ayudas para contratos de formación en Investigación Rio Hortega. Instituto de Salud Carlos III.

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Disclosures:

We hereby verify that the manuscript has not been submitted or accepted elsewhere. All authors have contributed to, seen, and approved the final, submitted version of the manuscript.

The authors have no conflicts of interest to disclose.

Running title: Newborn screening, primary immunodeficiencies, TRECS, KRECS

Abstract

Background: Early diagnosis of primary immunodeficiency such as severe combined immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA) improves outcome of affected children. *T-cell-receptor-excision circles* (TRECs) and *kappa-deleting-recombination-excision circles* (KRECs) determination from dried blood spots (DBS) identify neonates with severe T- and/or B-lymphopenia. No prospective data exist of the impact of gestational age (GA) and birth weight (BW) on TRECs and KRECs values. **Methods:** TRECs and KRECs determination using triplex RT-PCR (TRECS-KRECS-β-actin-Assay) from prospectively collected DBS between 02/2014 and 02/2015 in three hospitals in Seville, Spain. Cut-off levels were TRECs<6/punch, KRECs<4/punch and -β-actin>700/punch. Internal (SCID, XLA, ataxia telangiectasia) and external controls (NBS quality assurance program, CDC) were included. **Results:** A total of 5160 DBS were tested. Re-punch was needed in 77 samples (1.5%) due to insufficient β-actin (<700 copies/punch). Preterm neonates (GA<37 weeks) and neonates with a BW<2500g showed significantly lower TRECs and KRECs levels (p<0.001). Due to repeat positive results five neonates were re-called (<0.1%): Fatal chromosomopathy (n=1; TRECs 1/KRECs 4); extreme prematurity (n=2; TRECs 0/KRECs 0 and

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TRECs 1/KRECs 20 copies/punch); neonates born to mothers receiving azathioprine during pregnancy (n=2; TRECs 92/KRECs 1 and TRECs 154/KRECs 3 copies/punch). All internal and external controls were correctly identified.

Conclusions: TRECS-KRECS--β-actin-Assay correctly identifies T- and B-cell lymphopenias. Prematurity and low BW is associated with lower TREC and KREC levels. Extreme prematurity and maternal immune suppressive therapy may be a cause for false positive results of TRECs and KRECs values, respectively. To reduce the rate of insufficient samples, DBS extraction and storage need to be improved.

Introduction

Severe T- and/or B-cell lymphopenias in the first days of life may indicate congenital immune defects such as X-linked agammaglobulinemia (XLA) and severe combined immunodeficiency (SCID) [1]. *Whilst the incidence of SCID is considered to be at least 1:35.000 births in some US states, the true incidence in Spain is unknown.* Although SCID and XLA belong to the group of rare diseases, their incidences of 1:35,000 to 1:200,000 live births is still considerably high given their severity, particularly in SCID patients [2,3,4]. The common lack of a suggestive family history and the absence of specific clinical symptoms in the first weeks of life often lead to a delayed diagnosis, associated with prolonged intensive care treatment, high morbidity and mortality [5-7].

To date, no European country performs newborn screening (NBS) for primary immunodeficiencies (PIDs) routinely, despite meeting most of the established screening criteria, including the existence of relatively non-invasive screening methodology, proven benefit of early diagnosis, presence of an initial asymptomatic period, high morbidity and mortality, availability of curative or effective therapeutic options and a favourable cost-benefit analysis [8-10].

In contrast to most of the diseases included in the routine NBS, curative treatment approaches for SCID patients exist in form of hematopoietic stem cell transplantation (HSCT)

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or gene therapy [11,12]. Furthermore, early diagnosis avoids preventable iatrogenic events related to live vaccines such as rotavirus [13,14]. In patients with congenital agammaglobulinemias, prompt diagnosis and immunoglobulin substitution have shown to improve overall outcomes [15].

The quantification of two circular DNA fragments (*T-cell-receptor-excision circles*, TRECs and *kappa-deleting-recombination-excision circles*, KRECs) in dried blood spots (DBS) and tandem mass spectrometry for enzyme defects associated with PID are currently available for the systemic immunodeficiency screening in newborns [16-19]. TRECs and KRECs are formed during the genetic rearrangement necessary for the adequate T- and B-lymphocyte maturation. These DNA circles exhibit a dilution pattern, as they are not replicated during mitosis. Thus, newborns with severe PIDs associated with T- and/or B-cell lymphopenias, present low TRECs and KRECs copy numbers, regardless of the molecular aetiology of the underlying PID. This is important given their broad genetic heterogeneity [16-20].

In the USA 32 states have implemented the systematic NBS for T-cell deficiencies using real-time PCR quantifying TRECs in DBS [21]. In Europe, research groups in France and the United Kingdom have tested this TREC based methodology in retrospective studies [22,23]. More recently, a multiplex-PCR has been developed to detect not only T-cell lymphopenias (e.g. SCID, through low levels of TRECs) but also B-cell lymphopenias (through low levels of KRECs) that are associated with other severe PIDs such as XLA or ataxia telangiectasia (AT). This technique was initially evaluated in a cohort of 2560 anonymized DBS in Sweden [18] and afterwards prospectively tested by our group in Seville [24].

Currently, no consensus exists regarding the optimal screening technique. Whilst TREC based methods have been broadly used in the USA [3], there are reports about missed SCID, which would have been identified with the TRECs/KRECs/ACTB multiplex-PCR or tandem mass spectrometry [25-26]. KRECs measurement increases the diagnostic accuracy for specific SCID phenotypes such as ADA-SCID and enables to identify other PID such as AT, Nijmegen-Breakage-Syndrome and inherited agammaglobulinemias [1,10,18,24]. However, some experts suggest, that the inclusion of KRECs may substantially increase the rate of false-positive results, leading to an unacceptable number of sample re-testing, creating unnecessary family anxiety, harm to healthy newborns and increased costs especially in the

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preterm neonate group [27].

In this article we present our experience with the TRECs/KRECs/ACTB-assay in the 1st year since its implementation providing data on the feasibility and performance and describe the incidence and aetiology of T- and B-cell lymphopenias.

Material and Methods

Study design, setting and population

On-going prospective, observational and longitudinal study to determine TRECs and KRECs levels in DBS, obtained from neonates born in three hospitals in Seville, Spain (annexe), between February 2014 and March 2015. The local ethics committees approved this study. Samples were included once the legal guardians signed informed consent forms.

Sample and data collection

Heel prick blood samples were dropped on Schleicher&Schuell filter paper between the 3rd and 5th day post-partum as part of the routine neonatal screening process. In case of prematurity (gestational age (GA) <37 weeks), heel pricks were repeated every two weeks until week 37 of corrected GA, birth weight (BW) ≥2500g or normality in the assay. Two 3.2mm discs were punched for each sample, and stored at 4°C until use. Demographic and clinical data including gender, GA, BW was collected.

Sample processing and multiplex real-time PCR (TRECs/KRECs/ACTB-assay)

Samples were processed and analysed as previously described [24]. Briefly, after DNA extraction from DBS samples and DNA purification (DNA Elution and Purification Solution, Qiagen, Maryland, USA) TRECs, KRECs, and β-actin (ACTB) copy numbers were determined. The amount of ACTB copy numbers indicates the efficacy of DNA extraction from DBS samples.

Quality controls

Internal controls (designed and obtained by plasmid cloning [18]) and 20 blinded external controls provided by the NBS Quality Assurance Program of the Centers for Disease Control and Prevention (CDC), USA were analysed. In addition, seven control samples obtained from

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previously diagnosed patients from four Spanish immunodeficiency units were included.

Definition and interpretation of results

Based on our previous experience the following cut-off scores for an estimated 99.8% sensitivity in detecting severe T- and/or B-cell lymphopenias were defined as following: TRECs<6/punch, KRECs<4/punch, ACTB>700/punch [22]. False-positive results were defined as values below the established cut-offs for TRECs or KRECs in absence of SCID or inherited agammaglobulinemia, respectively. In case of abnormal or inconclusive results, a new punch from the same Guthrie card was obtained and the PCR-assay repeated (*re-test*). Only pathologic results in the *re-test* led to the request of a new heel prick sample (*re-call*) and the confirmation of a result below the established cut-off was followed by an assessment of the newborn. In case of insufficient material of the 1st Guthrie card despite re-punching, re-call with extraction of a 2nd DBS was offered, but not routinely performed.

Data analysis

Qualitative variables are shown as absolute frequencies and percentages. Quantitative variables were tested (*Kolmogorov–Smirnov test*, or *Shapiro–Wilk test*) for normal distribution and are expressed as mean±standard deviation (SD) or as median and interquartile range (IQR), when appropriate. Rates for abnormal, inconclusive, and normal results in the TRECs/KRECs-assay were calculated (CI 95%). To assess the assay reliability, the proportion of false-positive results was calculated. For qualitative variables the *chi-squared* or the *Fisher´s exact test* were applied to estimate variable associations with T- and B-cell lymphopenias. For quantitative variables the *Student's t-test* for independent samples or the *Mann–Whitney U test* were performed. Correlations were assessed by means of the Pearson tests. *p*-values of <0,05 were considered as statistical significant. All statistical operations were performed using the IBM software SPSS Statistics version 20.

Results

Demographic and clinical data of the cohort

A total of 5279 samples were prospectively collected from 5160 neonates. 51.2% were males; 74.6% were born at term (GA ≥37 weeks) and 79.3% had a BW ≥2500g. The mean GA

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was 38.6 weeks (±2.3 weeks) and the mean BW was 3147g (±610g). Information regarding BW or GA was incomplete for 480 (9.3%) and 772 (15.0%) valid samples, respectively.

Sample quality and assay performance

Out of 5160 neonates, 109 showed low copies of ACTB; 32 of which could not be repeated due to insufficient material (0.6%). *Re-punch* of the remaining 77 samples (1.5%) revealed 10 positive results and were *re-called* (0.19%); five had subsequently normal results and the remaining five samples were confirmed to be positive (0.1%).

Influence of GA and BW on TRECs, KRECs and ACTB levels

Results of the TRECs/KRECs-assay, calculated for the subgroups of GA and BW, are shown in Table 1. TRECs and KRECs copy numbers were significantly lower in preterm neonates compared to term neonates ($p<0.001$). Similarly, newborns with a BW <2500g showed lower TRECs and KRECs copy numbers than those with a BW ≥2500g ($p<0.001$). A positive correlation between GA and BW with TRECs ($r=0.117$, $p<0.001$; $r=0.118$, $p<0.001$) as well as with KRECs ($r=0.05$, $p=0.001$; $r=0.052$, $p=0.001$) was observed. For both variables this association was more pronounced for the TRECs. No differences were found when analysing the effect of GA and BW on the ACTB copy numbers.

Positive results from prospectively tested DBS

As shown in Figure 1 and Table 2a five of the 5160 prospectively tested newborns showed positive results in the assay. Patient 1 (P1) was a preterm female diagnosed with a complex chromosomopathy. Confirmation of the initially positive results with a second sample or lymphocyte subsets (LSS) determination could not be performed due to the rapidly fatal course of this patient. Two other individuals (P2, P3) with positive results in the 1st test run were identified as blood product dependent extreme preterm neonates. Low ACTB and TRECs were found in both individuals. In addition P3 also showed reduced KREC copy numbers. All three patients had a low for age lymphocyte count whilst LSS distribution of patients 2 and 3 was normal. A 2nd sample of the later demonstrated normal KRECs and ACTB levels whilst TRECs remained reduced. Repeated LSS were subsequently normal for age in P3 but not available for P2. Both patients are currently under follow-up. Low KRECs were detected in P4 and P5. A telephone interview with the family revealed both mothers

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receiving immunosuppressive treatment for systemic lupus erythematosus (SLE) and Crohn's disease including azathioprine during pregnancy. Whereas the family of P4 refused to perform a second heel prick, P5 demonstrated persistent low KREC levels at one month of age. However, a 3rd blood sample taken from P5 four weeks after stopping breast-feeding showed a normal LSS and KREC levels (Table 2b). A simultaneous blood sample taken from the mother of P5 revealed B-cell lymphopenia (39 cells/µl) and pathological TRECs and KRECs copy numbers (0 and 1, respectively) whilst receiving azathioprine treatment.

External control samples of the CDC

Four sets of blinded samples (n=20) were provided for quality assessment from the CDC. All positive (low TREC copy numbers, β-actin above cut-off), and negative samples (TREC and β-actin above cut-off) were correctly identified as shown in Figure 1, as it was the case with DNA free samples (undetectable TREC/KREC/β-actin; not included in Figure 1).

Samples from patients with known PID

Seven samples from patients with known underlying PID were included (Table 2b). Although in all cases the first available sample was used for analysis, age variation was broad (0.2-93 months). Four patients suffered from SCID (age range 0.2-9.6 months). TREC levels in these patients were all below the cut-off (range 0-4 copies/µl). Similarly, KRECs were abnormal in two subjects with a T-B-SCID phenotype. Two patients with XLA were included, aged 60 and 93 months at first DBS, and KRECs were correctly absent. One patient with AT had absent TRECs and KRECs in the lower limit. All included samples showed normal ACTB levels.

Discussion

This is currently the largest prospective study in Europe using the triplex TRECs/KRECs/ACTB-assay for NBS of severe T- and B-cell lymphopenias. Previous studies with mainly retrospective design from Sweden, UK, France and Israel highlight the importance for its implementation in Europe [18,22,23,28]. The USA data showed the efficacy, necessity and cost-effectiveness of NBS for T-cell deficiencies [3,9,27]. No consensus exists regarding the best screening methodology. Specifically the addition of KRECs to the assay is still under debate [1,10,27]. Algorithms for structured sample

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processing, result interpretation and decisions regarding the clinical follow-up are unanswered but important issues for all screening centers [10,27].

In this ongoing study we prospectively collected and analyzed 5279 samples from 5160 newborns. Values of TRECs and KRECs were found to be similar to other previously studied populations [18]. The initially chosen cut-off levels for TRECs and KRECs were progressively optimized in order to avoid unnecessary sample drawing and analysis related with unjustified harm to the newborn and additional costs [24]. Despite this adjustment the *re-punch* rate remained high, being 1.5% whilst *re-call* rate was low being 0.19%, complying with previous studies (10). Poor pre-analytic sample quality, mainly due to inappropriately prepared DBS (insufficient blood quantity and storage) resulted in low ACTB and was the most frequently identified reason for *re-punching*. Implementation of training programs and optimized communication with primary care centers nursery are needed to improve routine NBS performance and subsequently *re-punch* and *re-call* rates. The close collaboration with the regional screening center enabled us to avoid additional harm to newborns with insufficient DBS. Only two newborns were re-called, the remaining 8 newborns being previously contacted by the metabolic screening center for routine NBS repetition. We found an association between TRECs and KRECs levels with the demographic variables GA and BW. Lower TREC values have been previously described in preterm neonates [3,23,29], whereas no reports exist on the influence of GA and BW on the KREC levels. Only two extreme preterm neonates admitted to the neonatal intensive care unit showed TREC levels below the established cut-off whilst none had pathological KREC levels, suggesting the addition of KRECs to the assay not to increase the number of false-positive results in the preterm population. Overall the impact of GA and BW on TRECs was stronger compared to the KRECs. This is in line with a recent study investigating the maturation of the human immune system at different gestational ages, demonstrating B-cell development to precede T-cell maturation in human fetuses [30].

Two term neonates had KRECs below the cut-off with normal TREC values. Both neonates were born to mothers receiving immunosuppressive therapy during pregnancy with azathioprine for SLE and Crohn’s disease, highlighting the importance of a complete clinical history as part of the second tier testing. The potential association of immunosuppressive therapy and abnormal KRECs but normal TRECs has not been previously described and may be important, not only in light of incremented rates of false-positive results but also in

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expanding knowledge regarding immune maturation. Noteworthy, a repeat lymphocyte LSS as well as the determination of KRECS copies showed subsequently normal results of patient 5 (P5). Importantly, P5 received breast milk when the 2nd blood sample was taken and this was associated with abnormal low KRECs copies. In contrast, KRECs copies were normal four weeks after stopping breast-feeding as was a repeat LSS (3rd blood sample), whilst the mother continued on azathioprine maintaining abnormal low KRECs copies and B-cell lymphopenia, suggesting a transitional toxic effect of azathioprine on KRECs. This observation might be associated with potential “false” low KRECs levels and unnecessary repeat blood sampling.

All external control samples from the CDC were correctly classified. Similarly, the seven DBS from previously diagnosed patients with PID with T- and/or B-cell lymphopenia would have been picked up with the assay and the chosen cut-offs, thereby supporting previous studies, who have shown the test to be reliable, relatively non-invasive and cost-effective making the nationwide implementation of screening for PID in Spain and other European countries attractive [10,31]. The purely technical costs of the in-house TRECs/KRECs/ACTB-assay used here are 1.76€/sample compared with 4.67€/sample of the commercial TREC assays used in the USA [9,24]. Low costs, the potential to detect and further characterize a broader spectrum of severe PID may favor the use of the TREC/KREC/ACTB-assay [1,8,10,18].

In summary, this study provides new data from the currently largest prospective cohort in Europe using the triplex TREC/KREC/ACTB-assay for NBS. It confirms the high diagnostic accuracy and robustness of the technique. TREC and KREC levels show a significant correlation and association between GA and BW in the Southern Spanish population. None of the preterm neonates showed isolated abnormal KRECs suggesting that the additional KREC determination would not increase *re-punching* and *re-testing* rates. The impact of immunosuppressive therapy during pregnancy on the results of KRECs remains to be established. Importantly, optimization of DBS preparation is needed in the future.

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Annexe: Unidad de Neonatología, Hospital Universitario Virgen del Rocío, Sevilla**,** Unidad de Pediatría, Hospital Virgen de Valme, Sevilla**,** Unidad de Neonatología, Hospital Universitario Virgen de Macarena, Sevilla.

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Table 1: Results of TREC/KREC/ACTB PCR-assay of DBS in relation to gestational age variables and birth weight

Gestational age	N	(%)	TRECs/punch	*p*	KRECs/punch	P	ACTB/punch
≤28	38	0.7	36 [17-88]	***<0,001***	32 [22-60]	**0,02**	1714 [1093-2521]
29-31	43	0.8	59 [40-81]	***<0,001***	33 [18-54]	**0,003**	1345 [888-1541]
32-36	459	8.0	85 [49-131]	***<0,001***	37 [20-64]	***<0,001***	1498 [1001-2230]
≥37	3848	74.6	96 [62-141]	1	46 [27-75]	1	1673 [1117-2440]
unknown	772	15	92 [61-136]	NA	47 [26-76]	NA	1649 [1088-2535]
Birth weight	**N**	**(%)**	**TRECs/punch**	***p***	**KRECs/punch**		**ACTB/punch**
<1000	28	0.5	30 [14-45]	***<0,001***	32[15-41]	**0,003**	1512[900-2118]
1000-1499	64	1.2	75 [39-107]	***<0,001***	32 [20-55]	**0,001**	1434 [1140-2222]
1500-2499	496	9.6	87 [54-125]	***<0,001***	37 [21-60]	***<0,001***	1526 [1032-2211]
≥2500	4092	79.3	95 [62-140]	*1*	46 [27-75]	1	1663 [1102-2441]
unknown	480	9.3	96 [62-148]	*NA*	49 [25-80]	NA	1728 [1142-2604]
TOTAL (5160)	***5160***	***100***	***93 [60-139]***		***45 [26-74]***		***1649 [1100-2430]***

p values were calculated comparing gestational age and birth weight of preterms using corresponding term neonates as the reference category; *NA* not applicable

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Table 2a) Summary of false positive results from prospective DBS

Patient	GA/ GENDER	1st TREC/KREC/ACTB (copies/ punch)	2nd+ TREC/KREC/ACTB+ (copies/ punch)	3rd TREC/KREC/ACTB (copies/ punch)	1st LSS++ (cells/µl)	2nd LSS (cells/µl)	3rd LSS (cells/ µl)	Observations Diagnosis
P1	32/ female	1/4/1852	NA	NA	Total 500 CD3+ NA CD19+ NA	NA	NA	Died age 1 month Trisomy, Monosomy (46,XX,der(7)t(7;15)(p22;q22)mat), Hydrops fetalis, Ebstein anomaly
P2	25/ male	0/0/4	4/8/7199	NI	Total 2184 CD3+ 1366 (63%) CD19+ 367 (17%)	NA	NI	Extreme preterm Blood product dependent
P3	26/ male	1/20/356	0/27/2592	NA	Total 2424 CD3+ 1484 (60%) CD19+ 498 (20%)	Total 7493 CD3+ 2454 (35%) CD19+ 4500 (57%)	NA	Extreme preterm Blood product dependent TOF, IVIG dependent FU
P4	Term/ female	92/1/2953	NA	NA	NA	NA	NA	Azathioprine during pregnancy*
P5	Term/ male	154/3/3589	61/1/863	75/50/938	Total 1700 CD3+ NA CD19+ NA	NA	Total 5090 CD3+ 3844 (73%) CD19+ 1188 (23%)	Azathioprine during pregnancy**

FU follow-up; *GA* gestational age; *IVIG* intravenous immunoglobulin; *LSS* lymphocyte subsets; *NA* not available or refused by guardians; *NI* not indicated; *TOF* Tetralogy of Fallot

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+2nd sample was taken at 2 weeks of life, ++1st LSS were determined within 2 weeks from time of DBS result

* Mother diagnosed with SLE, not breast feeding; ** Mother diagnosed with Crohn´s disease, breast feeding until 4 weeks prior to 3rd blood sample was taken

Table 2b) Summary results from internal true positive controls

Control	AGE at Time of DBS (months)	Gender	TREC (copies / punch)	KREC (copies / punch)	ACTB (copies / punch)	LSS (cells/µl) 1st available sample	Diagnosis
C1	9.6	Male	0	56	705	Total 611 CD3+ 30 (4,7%) CD19+ 428 (67,1%)	T-B+SCID (undefined)
C2	0.2	Male	0.5	0	1257	Total 11800 CD3+ 9440 (80%) CD19+ (<1%)	T-B-SCID (RAG)
C3	0.2	Female	4	0	722	Total 300 CD3+ 79 (45%) CD19+ 1 (2%)	T-B-SCID (ADA)
C4	0.2	Male	1.9	0.6	1182	Total 1740 CD3+ 1087 (78%) CD19+ 1 (0,1%)	T^{low}B-SCID (Omenn)
C5	93	Male	23	0	3229	Total 3400 CD3+ 2598 (76%) CD19+ 2 (0,05%)	XLA
C6	60	Male	42	0	1980	Total 5594 CD3+ 5346 (92,9%) CD19+ 4 (0,1%)	XLA
C7	20	Male	0	7	805	Total 1678 CD3+ 664 (42,9%) CD19+ 314 (20,2%)	Ataxia telangiectasia

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