Research Article

Biomarkers in Medicine

Reference ranges of lymphocyte subsets in healthy Qatari adults

Aim: Determine reference ranges of lymphocyte subsets in healthy Qatari adults. Patients & **Methods:** Blood samples (n = 150) were investigated using four colors flow cytometery. **Results:** The mean percentage and absolute values of the lymphocyte subsets were: CD3: 73.6 ± 6.1%; 2,072 ± 644 cells/μl; CD4: 41.1 ± 7.9%; 1,167 ± 396 cells/μl; CD8: 33.8 ± 7.1%; 967 ± 364 cells/μl; B cells (CD19+): 11.3 ± 3.9%; 318 ± 144 cells/μl and natural killer (NK) cells (CD16/56+): 16.5 ± 6.3%, 440 ± 401 cells/μl, respectively. The CD4/CD8 ratio was 1.3 ± 0.5. The ratio, CD3+ and CD4+ percentage and CD4+ cell counts were significantly higher in females; CD8+ and NK cell count and CD8+ percentage were significantly higher in males. The impact of age was less certain. **Conclusion:** The reference values of lymphocyte subsets appear to be specific to this population, demonstrating the importance of establishing local reference ranges.

Keywords: B cells • CD3 • CD4 • CD8 • flow cytometry • lymphocyte subsets • natural killer cells • reference ranges,

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Introduction

Lymphocyte immunophenotyping by flow cytometry is an important tool in differential diagnosis of congenital and acquired immune deficiencies, lymphoproliferative disorders and acute leukemias [1]. In addition, it is used for monitoring treatment response and disease progression with anti-retroviral and immunosuppressive therapy. Various studies suggest that lymphocyte subsets reference ranges are influenced by age, sex, ethnicity, and environment [2–5]. The variations in the values of the lymphocyte subsets emphasize the importance of establishing local reference ranges to allow for meaningful comparisons and accurate interpretation of immunophenotyping data across different cultures and ethnic groups. Lifestyle factors, e.g. smoking, have also been shown to influence lymphocyte subset values [6,7].

Indeed, reference range for peripheral blood lymphocyte subsets have been established in many studies across different countries, revealing variations in the normal range for lymphocyte subsets according to age, sex, race, and environmental factors [3,4,8–16]. Establishment of an internal normal range of lymphocyte subsets is a important for each population considering the effect of environmental factors, age, ethnical diversity on the immune system. Hence, this study aims to determine the laboratory reference ranges for lymphocyte subsets in healthy Qatari adults using flow cytometry.

This article reports the local reference range for peripheral blood lymphocyte subsets, both percentages and absolute counts, in a representative sample of 150 healthy adult Qatari population.

The results have been stratified by age, sex, and smoking status. The population selected is largely homogenous (100% Qatari Arabs) so effect of race was not investigated. The results are briefly compared with other population data from published studies.

Materials & methods

Subjects

A total of 150 healthy Qatari volunteers (18–55 yr old; all Qatari Arabs) were recruited for the study from September 2013 to March

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2014. The authors aimed to recruit a maximum of 150 for this study, is realistic goal over the study period in a total population of approximately 300,000. The subpopulation evaluation was not powered to determined statistical differences due to the low number of the overall study population so the results would have to be interpreted with caution.

The Participants were screened initially with a health questionnaire that sought to identify and exclude those who had recent infections, recent vaccinations (within the past 4 weeks), any significant medical illnesses, or use of long-term medications. Subjects were also required to have their complete blood cell count (CBC) checked using the Beckman Coulter Hematology Analyzer. Participants with abnormal CBC participants were excluded from the study. Normal CBC ranges constituted: white blood cells (WBC) count 4–10 x109/l; hemoglobin range in men: 13–17g/dl, and for women: 11.5- 15 g/dl; platelets 150–450 ×109/l.

A signed consent form was obtained from all participants.

Blood collection

Whole blood was collected into EDTA Vacutainer tubes. The blood samples were transported to the laboratory and stained using BD Multitest IMK Kit with BD Trucount™ tubes (Cat. No. 340504, BD Bioscience, San Jose, CA, USA) within 24 h of collection. Samples were checked for clots or hemolysis formation before analysis.

Flow cytometry analysis

Lymphocyte subsets were analyzed on BD LSRFortessa™ flow cytometer (BD Bioscience). Antibodies to identify the lymphocytes were chosen to include the following: FITC-labeled CD3, CD8 phycoerythrin (PE), CD45 peridinin chlorophyllprotein (PerCP), and CD4 allophycocyanin (APC); PE-labeled CD16, and CD56, PerCP-labeled CD45 and APC-labeled CD19.

Gating the lymphocytes was based on CD45 versus side scatter analysis (SSC); and lymphocyte subpopulations were further analyzed from within this gate. The percentages of T and B lymphocytes were determined using quadrant statistics from dot plots of CD8 versus CD4 for suppressor/cytotoxic (CD3+ CD8+) and helper/inducer (CD3+ CD4+) T-lymphocytes; and CD16 + CD56 vs CD19 dot plots for B (CD19+) and natural killer (NK) cells (CD3– CD16+ or CD56+ or both) cells.

For the absolute number, a known volume of the sample was stained directly in a BD Trucount tube. The lyophilized pellet in the tube dissolved, releasing a known number of fluorescent beads. The absolute number (cells/μl) of positive cells in the sample was then determined by comparing cellular events to bead events using the following formula:

Absolute Cell Count = (number of target population events / number of bead events collected) × (number of beads per test from the package insert / test volume of 50 μl)

The percentage was calculated from the percentage of positive cells per lymphocyte population.

BD Multi-Check™ control (BD Catalog Nos. 340911) and the BD Multi-Check CD4 Low control (BD Catalog Nos. 340914) were used as procedural controls and were processed similarly to the test samples to monitor the outgoing performance of the analytic process. A performance check (CS&T) application in BD FACSDivaTM software was running using BD™ Cytometer Setup and Tracking (CS&T) Bead to monitor performance on a daily basis and to optimize laser delay

Statistical analysis

Data were analyzed using BD FACSDiva™ software 6.0. The results were reported as the percentage of positive cells per lymphocyte population or as the number of positive cells per microliter of blood (absolute count). The absolute numbers were not compared.

All data was analyzed using StatPlus® statistics software version 5.8.0.0. The distribution of T-cell subsets was compared between groups using the student t- test and p- values of <0.05 were considered significant.

Table 1. Baseline demographics of the the study population (n = 150)†.

Total (n)	150
Median Age	29 (range; 18–55 years)
Males	96
Females	54
Smokers	36
Non-smokers	114
≤45 years	135 (91 males; 44 females)
<25 years	43 (33 males; 10 females)
25–35 years	57 (45 males; 12 females)
35–45 years	35 (11 males; 24 females
≥45 years	15 (5 males; 10 females)

†All subjects were Qatari Arabs.

Results

Subjects

A total of 150 healthy Qatari adults ranging in age from 18 to 55 years were included in the study. The baseline demographics of the subjects are shown in Table 1.

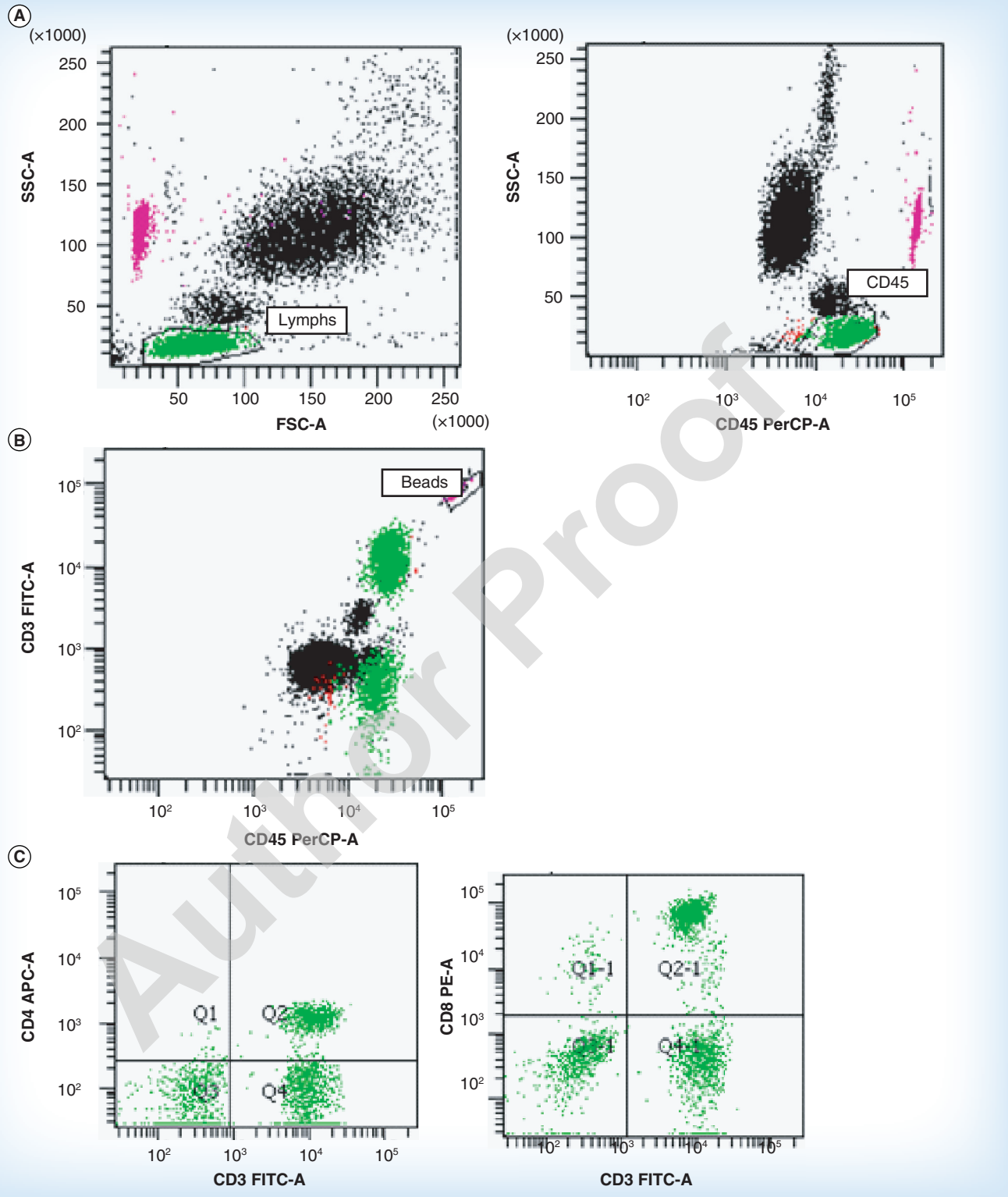

Figure 1. Examples of dot plots of flow cytometry data.(A) The CD45 vs SSC dot plot. The Gated lymphocyte population appeared as a bright, compact cluster with low SSC. Monocytes and granulocytes should also appeared as distinct clusters. **(B)** Gating of BD Trucount bead to identify BD Trucount absolute count bead events. **(C)** CD3 vs CD4 plot (left figure) and the right figure CD3 vs CD8, where Q2 is the percentage of CD3+CD4+ cells and Q2–1 is the percentage of CD3+CD8+ cells. **(D)** Dentification of suppressor/cytotoxic (CD3+CD8+) (Q1–2) and helper/inducer (CD3+ CD4 +) (Q4–2)lymphocytes in CD8 vs CD4 dot plot. **(E)** CD3 vs CD19 plot (left figure) and the right figure CD3 vs CD16+CD56, where Q1 is the percentage of CD3-CD19+ (Bcells) and Q1–1 is the percentage of CD3-CD16+CD56+ (natural killer cells). **(F)** Identification of B (CD19+) and NK (CD3–CD16+ or CD56+ or both) lymphocytes in CD16 + CD56 vs CD19 dot plot.
SSC: Side scatter.

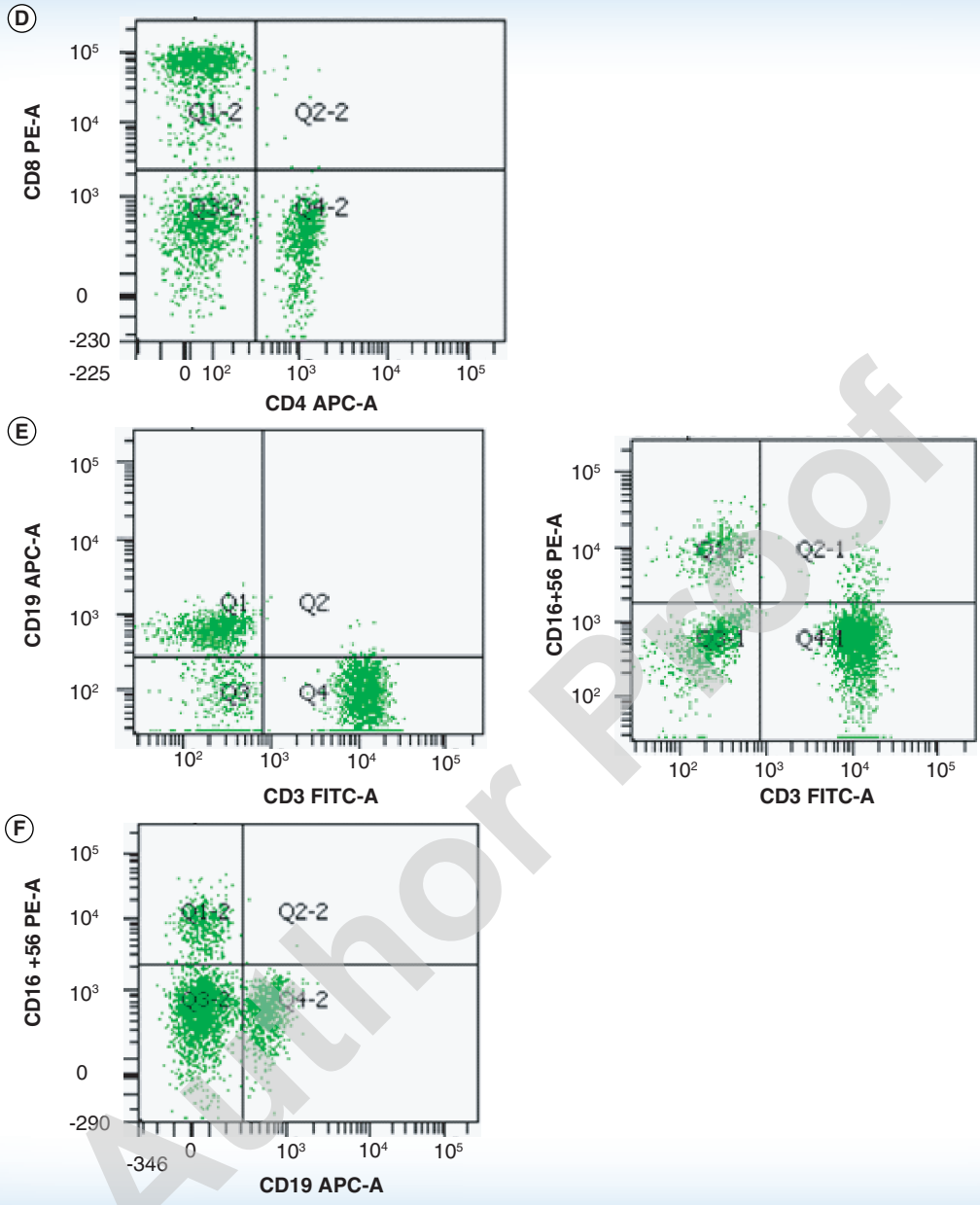

Figure 1. Examples of dot plots of flow cytometry data (cont.).(A) The CD45 vs SSC dot plot. The Gated lymphocyte population appeared as a bright, compact cluster with low SSC. Monocytes and granulocytes should also appeared as distinct clusters. **(B)** Gating of BD Trucount bead to identify BD Trucount absolute count bead events. **(C)** CD3 vs CD4 plot (left figure) and the right figure CD3 vs CD8, where Q2 is the percentage of CD3+CD4+ cells and Q2–1 is the percentage of CD3+CD8+ cells. **(D)** Dentification of suppressor/cytotoxic (CD3+CD8+) (Q1–2) and helper/inducer (CD3+ CD4 +) (Q4–2)lymphocytes in CD8 vs CD4 dot plot. **(E)** CD3 vs CD19 plot (left figure) and the right figure CD3 vs CD16+CD56, where Q1 is the percentage of CD3-CD19+ (Bcells) and Q1–1 is the percentage of CD3-CD16+CD56+ (natural killer cells). **(F)** Identification of B (CD19+) and NK (CD3–CD16+ or CD56+ or both) lymphocytes in CD16 + CD56 vs CD19 dot plot. SSC: Side scatter.

Flow cytometry

Figure 1 summarizes examples of dot plots of flow cytometry data. Total T cells were selected through CD3+ expression; T cells (CD3+) were then divided into CD4+ (helper;) and CD8+(cytotoxic) populations; NK cells and B cells were also selected through expression of CD16/56+ and CD19+, respectively. The absolute and percentage values of these cells were then determined. Table 2 demonstrates the reference ranges of lymphocyte subsets of the study population (n = 150).

When the reference ranges found in this study were compared with the reference ranges of the BD Multitest IMK Kit, significant differences were found (Table 3).

Table 2. Reference ranges of lymphocyte subsets in the healthy adult Qatari population (n = 150).

Cell type	Percentage % mean ± SD	Reference range 95% CI	Absolute cell count / µl mean ±SD	Reference range 95% CI
CD3+	73.6 ±6.1	56.5–92.6	2072 ± 644	792–3722
CD4+	41.1± 7.9	22.7–61.8	1167± 396	546–2591
CD8+	33.8± 7.1	19.8–55.5	967 ± 364	375–2532
B cells	11.3± 3.9	1.7–23.9	318 ± 144	43–750
Natural killer cells	16.5± 6.3	4.4–35.1	440 ± 401	103–1194
CD4/CD8 ratio (range)	1.3 ± 0.5 (0.4 -3.3)			

Effects of sex & age

The data were further analyzed according to sex and age. The reference ranges for males and females are tabulated in Table 4. The percentage of CD3+ T cells was significantly higher in females compared with males (75.3 vs 71.9%; $p < 0.001$); The percentage and absolute count values of CD4+ cells of females were also significantly higher compared with males (45.5 vs 38.0%, $p = 0.000$; 1256 cells/µl vs1130 cells/µl, $p \leq 0.05$, respectively). The percentage and absolute count of CD8+ cells were significantly higher in males compared with females (35.6 vs 30.8%, $p < 0.001$; 1079 cells/µl vs. 855 cells/µl, $p < 0.001$, respectively). The absolute number of NK cells was significantly lower in females compared with males (403 cells/µl vs 514 cells/µl; $p < 0.02$). There were no significant differences in the percentage and absolute count of B cell between males and females. The CD4/CD8 ratio was significantly higher in females compared with male ratios (1.1 vs 1.5; $p < 0.0001$) (Table 4).

In terms of age, 15 subjects were ≥45 years and 135 were ≤45 years old. Comparison of the reference ranges of lymphocytes subsets between the two groups is summarized in Table 5.

The percentage values of CD3+ and CD4+ cells as well as CD4/DC8 ratio were significantly increased in ≥45 years old group compared with ≤45 years old group. Conversely, there were significant decreases in absolute count of CD8+ and in absolute count and percentage of B cells in the ≥45 years old group comparing with ≤45 years old group. However, No significant differences in the NK cells between the two groups.

Effect of smoking

The lymphocyte subset percentages and absolute values were compared between smokers (n = 36) and non-smokers (n = 114). There were no significant differences in the percentages or absolute count values of lymphocyte subsets between smokers and non-smokers (data not shown). When this analysis was applied to just the male population (as there were no female smokers in our study), the same non-significant results were obtained (data not shown).

Discussion

In this study, we aimed to obtain reference ranges for lymphocyte subsets representative of the adult population in Qatar. The reference ranges identified are applicable to patients between 18–55 years of age. As far as we know, this is the first study that uses the flow

Table 3. Comparison of lymphocyte Subsets percentage between BD MultiTEST reference range and the reference range of the present study.

Cell type	Present Study (n = 150)		Representative reference ranges for BD Multitest (15)†(n = 164)		P value
	Mean (%)	Reference range (%)	Mean (%)	Reference range (%)	
CD3	73.6	56.7–92.6	72	56 - 86	NS
CD4	41.1	22.7- 61.8	45	33 - 58	<0.0001
CD8	33.8	19.8- 55.5	24	13- 39	<0.0001
B cells	11.3	1.7- 23.9	14	5–22	<0.0001
NK	16.5	4.4- 35.1	13	5–26	<0.0001

The reference ranges for BD Multitest were determined at three clinical investigation centers in the United States. Subjects were hematologically normal adults between the ages of 18 and 65 years.
NS: Non-significant.

Table 4. Lymphocyte subset percentages and absolute-number reference ranges for males and females in the study population (n = 150).

Cell type	Male (n = 96)		Female (n = 54)		P value	
	Mean absolute count (95% range)	Mean percentage % (95% range)	Mean absolute count (95%range)	Mean percentage % (95% range)	Absolute count	% percentage
CD3+	2196 (1181–3993)	71.9 (56.8–92.6)	2060 (793–3582)	75.3 (56.7–86.9)	NS	<0.001
CD4+	1130 (546–2135)	38.0 (21.2–64.1)	1256 (548–2592)	45.5 (28–61.8)	≤0.05	<0.00001
CD8+	1079 (376–2532)	35.6 (19.3–58.9)	855 (300–1813)	30.8 (19.8–41.6)	= 0.001	<0.001
CD19+	336 (43.2–728)	11.5 (1.7–24.3)	319 (103–750)	11.1 (5.1 -23.9)	NS	NS
Natural killer cells	514 (148–2383)	17.1 (6.0–44.0)	403 (103–1195)	15.0 (4.4–32.3)	<0.02	NS
CD4/CD8 ratio	1.2 (0.37–3.3)		1.5 (0.70–2.8)		<0.0001	

Absolute count (cells/μl); NS: Not significant.

cytometry method to establish lymphocyte subsets reference ranges in the Qatari population

Applicable flow cytometric analysis depends on the of reference values, which are subject to variation depending on various factors such as age and sex [17,18]. This variation suggests that each population should have its own lymphocyte subset reference ranges. Establishment of local reference ranges is important for interpretation of laboratory results [19].

In this study, the mean absolute number and percentage value of CD4+, CD8+, CD 19+ (B cells) and NK cells were significantly higher than the reference ranges of BD Multitest IMK Kit, suggesting a higher range for the Qatari population compared with the American Caucasian population [15].

Furthermore, the results obtained from our population were found to be different from that of other populations studied from published literature (Table 6). The absolute count of CD3, CD4, CD8 and NK cells in the Qatari adult population was markedly higher compared with those in the published studies. However, there appeared to be little difference in the absolute count of CD19 cells in our study compared with Kuwaiti, Dutch and Chinese studies. Overall, the Kuwaiti population study had reference ranges most comparable to our study (though there was still a large difference seen). This further confirms that each population does indeed need to establish their own reference ranges. Table 6 summarizes the comparison between lymphocyte subsets in this study compared with other population data.

It is important to note here that the Qatar population was composed predominately of young individuals (≤45 yr). The frequencies of lymphocytes in the older subjects in the Qatar group are more similar to the reference ranges reported in the literature than the younger Qatar subjects. The variation observed in this study may be accounted by the fact that here we stud-

Table 5. Lymphocyte subset percentages and absolute-number reference ranges for ≤45 yr and ≥45 yr old in the total study population (n = 150).

Cell type	≥45 years (n= 15) Mean (95% Range)		≤45 years (n= 135) Mean (95% range)		P value	
	Absolute count (range)	% Percentage (range)	Absolute count range)	% Percentage (range)	Absolute count	Percentage (%)
CD3+	1900 (1160–2704)	77 (60.3–92.6)	2173 (793–3993)	72.7 (56.7–86.9)	NS	<0.02
CD4+	1227 (848–1596)	48.8 (36.6–64.1)	1169 (546–2592)	39.8 (21.2–61.8)	NS	<0.0001
CD8+	781 (428–1293)	30.6 (19.3–42.9)	1022 (300–2532)	34.3 (19.8–58.9)	<0.05	NS
CD19+	213 (43–495)	8.5 (5.5–24.3)	341 (51–772)	11.8 (5.5 -24.3)	<0.01	<0.01
NK	386 (183–802)	15.9 (7.3–32.3)	484 (103–2382)	16.4 (4.4–44.0)	NS	NS
CD4/CD8 ratio	1.7 (1.0–3.3)		1.3 (0.4–2.8)		<0.01	

Absolute count (cells/μl); NS: Not significant

Table 6. Comparison of lymphocyte subsets reference range of the present study with other reference ranges.

Qatar (n=150)	CD3+	CD4+	CD8+	CD19+	Natural killer cells
Absolute count/ µl					
Mean± (SD)	2142 (676)	1165 (396)	967 (364)	319 (144)	498 (401)
Median	2009	1109	940	307	411
95% Range	792-3991	546-2591	300-2532	43-771	103-2382
Percentage%					
Mean± (SD)	73.2(6.5)	40.6 (8.1)	33.9 (7.6)	11.5 (4.3)	16.4 (7.0)
Median	73.6	40.2	33	10.7	15.1
95% Range	57-92.6	21.2-64.1	19.3-58.9	1.7-24.3	4.4-44
Kuwait (22) (n=127)					
Absolute count/ µl‡	1779 (470)	1059 (300)	739 (220)	339 (140)	329 (130)
Oman (40) (n= 50)					
Percentage†	76.5(8)	45 (6.8)	29.5 (6)	14 (4.3)	7 (4.3)
Caucasian (12) (n= 271)					
Percentage%*	73 (6.2)	43 (7.5)	33 (7.5)	14 (4.2)	14 (6.1)
Dutch (41)§ (n= 1365)					
Absolute count/ µl‡	1525 (458)	993 (319)	506 (220)	313 (147)	NA
Switzerland (42) (n= 70)					
Absolute count/ µl†	1075 (313)	691 (208)	347 (172)	170 (97)	183 (116)
Ethiopia (41) (n=142)					
Absolute count/ µl‡	1555 (463)	775 (225)	747 (333)	191 (94)	250 (137)
China (43) (n=273)					
Absolute count/ µl†	1362 (382)	760 (233)	515 (192)	298 (124)	229 (132)

Values given are mean ±(S.D).
Values given are median±(S.D).
Percentage values not available.
Dutch samples were controls in the Ethiopian study cited.
NA: Not available; NS: Not significant.

ied younger subjects than were studied in the reference ranges reported in the literature. A larger study with more balanced numbers of individuals under and over 45 years of age is needed to verify this.

In our study, significant increases were observed in the mean absolute numbers and percentages of CD3+ and CD4+ cells in females compared with males. Conversely, there were increases in the mean absolute number and percentage of CD8+ and NK cells in males compared with females. In the literature, some studies have reported higher values of CD3+ and CD4+ cells in females compared with males [2,11,12,16,20], while other studies report no difference between the two sexes [11,21,22]. Significantly lower NK values have also been reported in women vs. men [11,23].

The differences observed may be the result of hormonal differences between genders as seen in studies in mice [24,25]. The mechanism may involve androgens' effect on thymocytes, mediating the process of thymocyte selection, with the potential to impart gender-specific characteristics on the peripheral T cell repertoire [25,26].

While variable results have been reported in humans and mice regarding levels of NK and B cells, most of these studies described declining levels of these cells with aging [27–31]. With respect to T-cells, however, although some studies have reported changes in T cell levels, especially a decrease in the CD8 cells with increasing age [2,10,11,32–34], other (older) have reported increase in CD4+ and /or CD8+ [24,35,36]. The impact of age on lymphocyte subsets is not well established, with contradictory results from various studies. The inconsistency across published literature may be due to the fact that different studies compared different age

ranges of subjects. In our study we observed significant changes of T lymphocytes as well as B cells with ageing. Our data showed significant increase in CD3+, CD4+ and CD4/CD8 ratio with increasing age. Conversely, there were decreases in CD8+ and B cells with increasing age. There were no significant age-related changes in NK cells. However, in our cohort the majority of subjects were under 45 years of age. As such the impact of age cannot be fully elucidated and it may be worth looking at the data by various age ranges in a larger cohort to understand the effect of ageing on lymphocytes.

Contradictory findings have been reported by various studies regarding smoking and lymphocyte subsets [37–39]. Our study demonstrated no significant differences in lymphocyte subsets between smokers and non-smokers.

Conclusion

In conclusion, we have established local reference ranges for lymphocyte subsets for healthy Qatari adults in this study. The differences observed in lymphocyte subsets between our population and other studies may be the result of genetic, lifestyle and/or environmental factors, necessitating the establishment of population-based reference ranges. Our study further confirms the gender difference in lymphocytes. The impact of age was less certain.

Future perspective

The lymphocyte references ranges are very poorly established in Qatar as well as the rest of the Arab world. In the coming decade, we hope to:

- Replicate the results in a larger cohort of the Qatari population;
- Collaborate with other researchers in the Middle East to establish reference ranges for the entire Arab population;
- Establish the effect of age on lymphocyte ranges in a larger cohort stratified by age.

Hopefully, modification of gating strategies (especially development of automated gating) over the next few 5–10 years will allow for more robust and reliable data. Development of systems where multiple parameters can be analyzed at the same time can further help improve quality of data in this field.

Acknowledgements

A Al-Thani designed this study. W Saleem performed data analysis and drafted the manuscript. A Alnaqdy assisted in designing the study and drafted the study proposal. A Al-Marwani and H Sharafeldin helped collect the samples. All authors read and approved the final manuscript.

Financial & competing interests disclosure

This work was supported by a grant from Qatar University. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Writing assistance was utilized in the production of this manuscript. The authors would like to acknowledge support of S ati, from Medcommz Ltd in providing editorial support for this article. The authors wish to thank M Alkhinji and F Dadbakhash for technical assistance.

Ethical conduct of research

Hamad Medical Research Center Ethics Committee provided the ethical approval for this study. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive Summary

- We aimed to determine the reference ranges of lymphocyte subsets in healthy Qatari adults.
- Flow cytometery was used to establish normal reference range for peripheral blood lymphocyte in 150 adults.
- The mean percentage and absolute values of the lymphocyte subsets were found to be specific to this population and markedly different from reference ranges (based on the Western population)
- Significant differences between males and females were found in the percentage and cell counts of lymphocytes
- The impact of age was less certain.
- Our study demonstrates the importance of establishing local reference ranges for each population.

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