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ORIGINAL ARTICLE

SCCA and CYFRA 21-1 Reference Intervals for Apparently Healthy Chinese Adults: a Multicenter Cross-Sectional Study

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SUMMARY

Background: **This study aimed to establish reference intervals for two biomarkers actively utilized in routine annual medical check-ups in China: squamous cell carcinoma antigen (SCCA) and cytokeratin 19 fragment (CYRFA 21-1), and to understand the influence of age, gender, and benign nodule(s) on their levels.**
Methods: **This prospective multicenter cross-sectional study continuously enrolled apparently healthy adults attending annual medical check-ups at three sites in 2019. Serum SCCA and CYFRA 21-1 levels were measured using electrochemiluminescence immunoassays. Age- and gender-specific reference intervals for the two biomarkers were established by using the 0 - 95th percentiles with 90% confidence intervals (CIs). The 97.5th percentiles were also provided.**
Results: **A total of 1,017 subjects were enrolled in this study. Both biomarkers were significantly lower in females, and age was negatively associated with SCCA while positively associated with CYFRA 21-1 (all $p < 0.0001$). No statistically significant differences were determined between subgroups without/with benign nodule(s) despite nodule(s) status (all $p > 0.05$). The overall reference interval for SCCA is 0 - 2.64 ng/mL and 0 - 4.39 ng/mL for CYFRA 21-1. The age-specific reference intervals for SCCA are 0 - 2.76 ng/mL (18 - 49 years) and 0 - 2.22 ng/mL (≥ 50 years), and for CYFRA 21-1, they are 0 - 3.86 ng/mL (18 - 49 years) and 0 - 4.89 ng/mL (≥ 50 years). The gender-specific reference intervals for SCCA are 0 - 2.83 ng/mL (male) and 0 - 2.49 ng/mL (female), and for CYFRA 21-1, they are 0 - 4.34 ng/mL (male) and 0 - 4.45 ng/mL (female).**
Conclusions: **The reference intervals for SCCA and CYFRA 21-1 established in this study could be utilized in annual medical check-ups and contribute to the screening of lung cancer in China.**
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KEYWORDS

SCCA, CYFRA 21-1, reference interval, lung cancer screening, Chinese adults, medical check-up

INTRODUCTION

Early detection is widely recognized as an effective strategy for easing the burden of lung cancer, as most cases are initially asymptomatic and typically diagnosed at advanced stages [1] where effective treatments are challenging. Low-dose computed tomography (LDCT) screening has been actively used for this purpose, as it has been shown to reduce lung cancer mortality by 20% [2]. However, due to the aforementioned challenging national conditions in China and the high false positive rate of potential lung cancer in health check-ups arising from the lack of identifying high-risk populations [3], LDCT alone may, from the perspective of many physicians in China, not be sufficient. Therefore, many clinics have used biomarker screening, including squamous cell carcinoma antigen (SCCA) and cytokeratin 19 fragment (CYRFA 21-1), with LDCT, though clinical guidelines and consensus statements have not yet recognized its medical value. A recent study showed the superiority of combining LDCT and a plasma-based biomarker, a micro-RNA signature classifier, compared to net LDCT in China [4], which we think will increase discussions on biomarkers screening for early detection of malignancy.

SCCA was isolated in 1977 from the squamous cell carcinoma (SCC) tissues of the uterine cervix and has been actively studied as a biomarker in diagnosing and monitoring SCC [5]. Several studies have suggested the potential of serum SCCA level as a biomarker for early detection of lung cancer [6]. CYFRA 21-1 is a c-19 fragment that is soluble in serum and may be a useful circulating tumor marker, with the primary indication for CYFRA 21-1 being to monitor the progression of non-small cell lung cancer (NSCLC) [7,8]. CYFRA 21-1 has shown reasonable specificity in identifying premalignant lesions from lung diseases, such as pneumonia, sarcoidosis, tuberculosis, chronic bronchitis, bronchial asthma, and emphysema [9]. Overall, SCCA and CYFRA 21-1 are reasonable candidates for biomarker screening of lung cancer.

To evaluate SCCA and CYFRA 21-1 in medical screening, it is essential for physicians and specialists in medical check-ups to know their appropriate reference intervals in healthy populations and set them as the basis. However, in many clinical laboratories in China, the reference intervals of cancer biomarkers reported by the manufacturer's instructions were directly applied, normally established based on non-Chinese ethnicity/race population. This practice may lead to errors due to ethnicity/race and geographical disparity [10-12]. Therefore, establishing Chinese-specific reference intervals is encouraged. Although several studies have measured SCCA and CYFRA 21-1 in healthy subjects, their values are mainly used as the control group [13,14], and relevant reference intervals, especially for SCCA, were limited.

This study aimed to establish appropriate SCCA and CYFRA 21-1 reference intervals for apparently healthy Chinese subjects. The secondary objective of this study is to investigate the influence of several factors, including age, gender, and benign nodules, on the level of the two biomarkers. Information on benign nodules was applied as they are commonly reported in healthy check-ups and can potentially interfere with the levels. Moreover, we also want to compare the screening performance of using the upper reference interval limit (95th percentiles) of SCCA and CYFRA 21-1 obtained in this study with that in the manufacturer's instructions.

MATERIALS AND METHODS

Study design

Figure 1 shows the flowchart of this multicenter prospective cross-sectional study. This study was designed to enroll ≥ 120 males, ≥ 120 females, ≥ 120 subjects aged 18 - 50 years, and ≥ 120 subjects aged ≥ 50 years to establish gender- and age-specific reference intervals. All subjects must be apparently healthy adults, and each site was required to contribute ≥ 40 subjects in the four subgroups to mitigate regional bias. The sample size setting of this study is explained in detail in the statistical analysis below. Subjects that attended annual medical check-ups in 2019 at three hospitals in different regions in China were initially screened in this study to meet the required sample size: (1) Cancer Hospital of Fudan University (site 1), Shanghai; (2) The First Affiliated Hospital of Guangzhou Medical University, Guangzhou (site 2); and (3) Fujian Provincial Hospital, Fujian (site 3).

In this study, benign pulmonary nodules were detected using LDCT and assigned to low-risk subjects based on the Guidelines for Management of Incidental Pulmonary Nodules Detected on CT Images, established by the Fleischner Society in 2017 [15]. Also, benign thyroid nodule(s) were assigned when the nodule(s) show(s) a benign pattern or did not meet the fine needle aspiration (FNA) size cutoff, which is fully in accordance with the 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer [16].

Eligibility criteria

The inclusion criteria of this study were: 1) aged ≥ 18 years; 2) identified as an apparently healthy subject that is not suspected of having any cancer at the end of the medical check-up.

The exclusion criteria are: 1) pregnant or breastfeeding; 2) impaired liver/renal function with abnormal serum creatinine: > 1.5 mg/dL (133 μmol/L) or < 0.5 mg/dL (44 μmol/L). 3) medical records of surgery; 4) liver cirrhosis; 5) pneumonia and/or thyroiditis; 6) medical records of cancer; 7) suspected as cancer patient based on the medical check-up results; 8) cutaneous disorders at active phase, especially psoriasis and lupus erythematosus.

Laboratory measurements

A minimum of 0.5 mL of residual venous blood of each participant was collected in gel separator tubes and frozen at -80°C for testing in one batch at the central laboratory of each site within 12 weeks. At the clinical laboratory, each tube was centrifuged for 10 minutes at 3,000 rpm to separate serum. Samples that were insufficient for analysis, clotted, refrozen, hemolyzed, lipemic, or icteric were excluded.

The Roche cobas e 601/602 system (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure serum SCCA and CYFRA 21-1 levels by electrochemiluminescence immunoassay (Elescys® SCCA, measuring ranges, 0.1 - 70 ng/mL; Elescys® CYFRA 21-1, measuring ranges, 0.1 - 500 ng/mL). All the reagents and instruments were used strictly according to the standard operating procedure (SOP) and package inserts provided by the manufacturer.

To minimize any potential bias related to pre-analytical sample handling, researchers at each site underwent additional training to ensure they were fully familiar with the functionality and handling details of the instruments and reagents used in this study. To further ensure accuracy in measurement and reliability of the results obtained, internal quality controls (IQC) were conducted at each site. The coefficients of variation (CVs) of the quality control assay for the two biomarkers were all ensured to be below 5% (as shown in Table S1) before measuring the serum samples.

Statistical analysis

The percentile method was used to establish a nonparametric reference interval, with lower and upper reference limits at a 90% confidence level. A minimum sample size of 120 subjects was required for this method, as established by the Clinical and Laboratory Standards Institute (CLSI) in 2008 as well as the Nation Health Commission of the People's Republic of China in 2012: Define and determine the reference intervals in clinical laboratory (Standard ID: WS/T 402 2012).

Data normality was tested by the Kolmogorov-Smirnov test. Quantitative variables were described as mean ± standard deviation (SD) or median with interquartile range (IQR). The study population was stratified into subgroups according to gender (male and female) and age (18 - 49 years and ≥ 50 years). Nonparametric Wilcoxon rank-sum tests were performed to compare the differences among different subgroups. Lower and upper reference interval limits were estimated by using the 0th and 95th percentiles. Because serum SCCA and CYFRA 21-1 levels are not normally distributed, distribution-free 90% confidence limits for the 2.5th, 95th, and 97.5th percentiles were computed by using order statistics (ranks). Besides, univariate and multivariate linear regressions were conducted by using SCCA or CYFRA 21-1 as the dependent variable and age and gender as the independent variable.

All the data in this study were analyzed using SAS 9.4 statistical software. $p < 0.05$ was determined as statistically significant.

RESULTS

Baseline demographic and clinical characteristics of the study population

This study enrolled 1,017 healthy subjects, including 618 females and 399 males. The mean (SD) age of the study population was 44.1 (14.2). Fudan University Shanghai Cancer Center, Shanghai (Site 1), contributed 39.3% (400/1,017) of the sample size. All participants had their serum SCCA and CYFRA 21-1 levels measured, with the median (IQR) of 1.23 (0.95 - 1.63) ng/mL and 2.19 (1.63 - 2.87) ng/mL, respectively. We also collected additional information on the 400 participants enrolled from site 1, including creatinine level and clinical information on benign nodule(s). The mean (SD) serum creatinine level (ng/mL) of these subjects was 68.64 (15.62), and 50.8% of the subjects had benign nodule(s), regardless of pulmonary, thyroid, or both.

Differences of SCCA and CYFRA 21-1 distributions among subgroups stratified based on age, gender, and benign nodule(s)

Based on the previous studies that established the influence of age and gender on the serum levels of SCCA and CYFRA 21-1, this study also investigated the distribution of each biomarker in age- and gender-specific subgroups (Figure 2). The demographic and clinical characteristics of subgroups stratified by age and gender are also demonstrated in Table 1.

Of the study population, 618 subjects were female and 399 were male. The serum level (ng/mL) of SCCA and CYFRA 21-1 were skew-distributed in both gender groups, giving the median (IQR) of 1.15 (0.86 - 1.51) for SCCA in females, 1.38 (1.08 - 1.83) for SCCA in male, 2.19 (1.63 - 2.87) for CYFRA 21-1 in female, and 2.41 (1.91 - 3.04) for CYFRA 21-1 in male. For both biomarkers, the serum level was significantly different between the two gender groups (both $p < 0.05$). When the study population was stratified by age, significant differences between the two age groups were also determined in the two biomarkers ($p < 0.05$). The median (IQR) serum level (ng/mL) of SCCA was 1.31 (1.04 - 1.75) ng/mL in the 18 - 49 years subgroup and 1.09 (0.84 - 1.51) ng/mL in the > 50 years subgroup. The corresponding value of CYFRA 21-1 was 2.15 (1.63 - 2.8) ng/mL and 2.47 (1.94 - 3.22) ng/mL, respectively. In the 400 subjects with available creatinine results, a positive and weak correlation was identified in both biomarkers (all $p < 0.0001$). The Spearman p-values were found to be 0.2606 for SCCA and 0.1963 for CYFRA 21-1, respectively.

To understand the influence of SCCA and CYFRA 21-1 distribution from benign nodule(s), this study stratified

Table 1. Demographic and clinical characteristics of the study population.

		Study population	Stratified by gender			Stratified by age		
			female (n = 618)	male (n = 399)	p	18 - 49 years (n = 619)	> 50 years (n = 398)	p
Age years	median (IQR)	42 (32 - 55)	41 (31 - 54)	45 (33 - 56)	0.0073	\	\	
	mean (SD)	44.1 (14.2)	43.3 (14.5)	45.4 (13.6)		\	\	
SCCA ng/mL	median (IQR)	1.23 (0.95 - 1.63)	1.15 (0.86 - 1.51)	1.38 (1.08 - 1.83)	< 0.001	1.31 (1.04 - 1.75)	1.09 (0.84 - 1.51)	< 0.001
	mean (SD)	1.39 (0.73)	1.29 (0.72)	1.54 (0.71)		1.49 (0.79)	1.22 (0.56)	
CYFRA 21-1 ng/mL	median (IQR)	2.26 (1.73 - 2.94)	2.19 (1.63 - 2.87)	2.41 (1.91 - 3.04)	< 0.001	2.15 (1.63 - 2.8)	2.47 (1.94 - 3.22)	< 0.001
	mean (SD)	2.45 (1.01)	2.38 (1.03)	2.56 (0.99)		2.28 (0.86)	2.71 (1.17)	
Creatinine µmol/L *	median (IQR)	66 (57 - 79.75)	59 (53 - 65.8)	82 (74 - 90)	< 0.001	63.8 (55 - 78)	70 (60 - 81.8)	< 0.001
	mean (SD)	68.64 (15.62)	59.62 (6.94)	82.58 (12.56)		66.78 (15.21)	71.53 (15.84)	
Clinical information on benign nodule(s)[1] n = 400								
Without benign nodule		203 (50.8)	137 (51.1)	66 (50)	0.8332	134 (53.0)	69 (46.9)	0.2452
With any benign nodule(s) n (%)[2]		197 (49.2)	131 (48.9)	66 (50)		119 (47.0)	78 (53.1)	
With pulmonary nodule(s) n (%)[3]		120 (30.0)	76 (28.4)	44 (33.3)	0.3073	56 (22.1)	64 (43.5)	< 0.001
With thyroid nodule(s) n (%)[4]		124 (31.0)	87 (32.5)	37 (28.03)	0.3674	83 (32.8)	41 (27.9)	0.3055
With benign pulmonary and thyroid nodule n (%)[5]		47 (11.8)	32 (11.9)	15 (11.4)	0.8663	20 (7.9)	27 (18.4)	0.0017

[1] Data obtained at Fudan University Shanghai Cancer Center. n = 400. The other two centers did not collect this information.
[2] Reference group: subjects without benign nodule(s).
[3] Reference group: subjects without pulmonary nodule(s).
[4] Reference group: subjects without thyroid nodule(s).
[5] Reference group: subjects without pulmonary and thyroid nodule(s) at the same time, including those with pulmonary or thyroid nodule(s) and those without any nodule(s).

the population into five subgroups (see Table 1). The subgroup for subjects without benign nodules had the median (IQR) SCCA of 1.26 (1.04 - 1.66) ng/mL and CYFRA 21-1 of 2.19 (1.66 - 2.73) ng/mL. As demonstrated in Table 2, there was no statistical significance in serum level of both SCCA and CYFRA 21-1 between subjects without and with benign nodule(s), regardless of its type, and the relevant reference subgroups were determined (all $p > 0.05$).

Establishing the reference interval for SCCA and CYFRA 21-1

For both SCCA and CYFRA 21-1, the overall reference intervals based on the whole population and a series of age- and gender-specific reference intervals were established in this study. Based on the standards and statistical methods applied, the ranges from 0th to 95th percentile should be recommended as the reference intervals for the two biomarkers for clinical practices in China. The percentile reference interval based on the whole study population for SCCA and CYFRA 21-1 (ng/mL) is 0 - 2.64 and 0 - 4.39, respectively. Specifically, the age-specific reference intervals for SCCA (ng/mL) are 0 - 2.76 (18 - 49 years) and 0 - 2.22 (≥ 50 years), as well as for CYFRA 21-1 (ng/mL), they are 0 - 3.86 (18 - 49 years) and 0 - 4.98 (≥ 50 years). The gender-specific reference intervals for SCCA (ng/mL) are 0 - 2.83 (male) and 0 - 2.49 (female), as well as for CYFRA 21-1, they are 0 - 4.34 (male) and 0 - 4.45 (female).

Table 2. Serum SCCA and CYFRA 21-1 levels in subjects with pulmonary and/or thyroid nodule(s).

Subgroups				p
No benign nodule(s) (n = 203)	SCCA	mean (SD)	1.41 (0.59)	
		median (IQR)	1.26 (1.04 - 1.66)	\
	CYFRA 21-1	mean (SD)	2.25 (0.79)	
		median (IQR)	2.19 (1.66 - 2.73)	\
Any benign nodule(s)[1] (n = 197)	SCCA	mean (SD)	1.42 (0.73)	
		median (IQR)	1.28 (0.99 - 1.72)	0.6195
	CYFRA 21-1	mean (SD)	2.42 (1.20)	
		median (IQR)	2.09 (1.66 - 2.89)	0.7994
Benign pulmonary nodule(s)[2] (n = 120)	SCCA	mean (SD)	1.42 (0.75)	
		median (IQR)	1.29 (0.92 - 1.7)	0.5935
	CYFRA 21-1	mean (SD)	2.45 (1.32)	
		median (IQR)	2.07 (1.62 - 2.83)	0.9846
Benign thyroid nodule(s)[3] (n = 124)	SCCA	mean (SD)	1.43 (0.73)	
		median (IQR)	1.28 (1.03 - 1.72)	0.7975
	CYFRA 21-1	mean (SD)	2.39 (1.17)	
		median (IQR)	2.06 (1.58 - 2.86)	0.7503
Benign pulmonary and thyroid nodule(s)[4] (n = 47)	SCCA	mean (SD)	1.43 (0.78)	
		median (IQR)	1.29 (0.91 - 1.73)	0.7211
	CYFRA 21-1	mean (SD)	2.41 (1.45)	
		median (IQR)	2.03 (1.45 - 2.63)	0.4101

[1] Reference group: subjects without benign nodule(s).
[2] Reference group: subjects without pulmonary nodule(s).
[3] Reference group: subjects without thyroid nodule(s).
[4] Reference group: subjects without pulmonary and thyroid nodule(s) at the same time, including those with pulmonary or thyroid nodule(s) and those without any nodule(s).

Table 3. Reference interval of SCCA and CYFRA 21-1 for healthy medical check-up population in China.

		2.5th percentile (90% CI)	50th percentile (90% CI)	95th percentile (90% CI)	97.5th percentile (90% CI)
SCCA	whole population	0.55 (0.53 - 0.58)	1.23 (1.2 - 1.26)	2.64 (2.49 - 2.81)	3.00 (2.85 - 3.33)
	Age-specific reference intervals				
	18 - 49 years	0.61 (0.57 - 0.65)	1.31 (1.27 - 1.34)	2.76 (2.64 - 2.87)	3.24 (2.93 - 4.71)
	≥ 50 years	0.48 (0.44 - 0.54)	1.09 (1.05 - 1.17)	2.22 (2.03 - 2.49)	2.82 (2.39 - 3.17)
	Gender-specific reference intervals				
	male	0.68 (0.57 - 0.71)	1.38 (1.32 - 1.45)	2.83 (2.61 - 3.08)	3.29 (2.93 - 4.52)
	female	0.54 (0.47 - 0.55)	1.15 (1.11 - 1.18)	2.49 (2.26 - 2.70)	2.85 (2.74 - 3.22)
CYFRA 21-1	whole population	1.06 (0.98 - 1.1)	2.26 (2.22 - 2.33)	4.39 (4.09 - 4.56)	4.93 (4.71 - 5.21)
	Age-specific reference intervals				
	18 - 49 years	1.02 (0.9 - 1.06)	2.15 (2.11 - 2.22)	3.86 (3.64 - 4.21)	4.51 (4.28 - 4.71)
	≥ 50 years	1.25 (1.07 - 1.31)	2.47 (2.38 - 2.6)	4.89 (4.45 - 5.21)	5.64 (5.11 - 7.1)
	Gender-specific reference intervals				
	male	1.13 (0.95 - 1.27)	2.41 (2.34 - 2.48)	4.34 (3.94 - 4.64)	4.96 (4.51 - 5.64)
	female	1.04 (0.93 - 1.07)	2.19 (2.13 - 2.24)	4.45 (4.12 - 4.66)	4.93 (4.72 - 5.32)

Table 4. Screening performance of the upper limit (95%th percentile) of the reference interval established in this study and the manufacturer's instruction.

	This study *	Manufacturer's instruction	False positive rate **
SCCA upper limit ng/mL	**2.64**	**2.3**	**7.96%**
CYFRA 21-1 upper limit ng/mL	**4.39**	**3.3**	**16.12%**

*** - Reference group.**
**** - The false positive rate of SCCA and CYFRA 21-1 screening in this study population is 5%.**

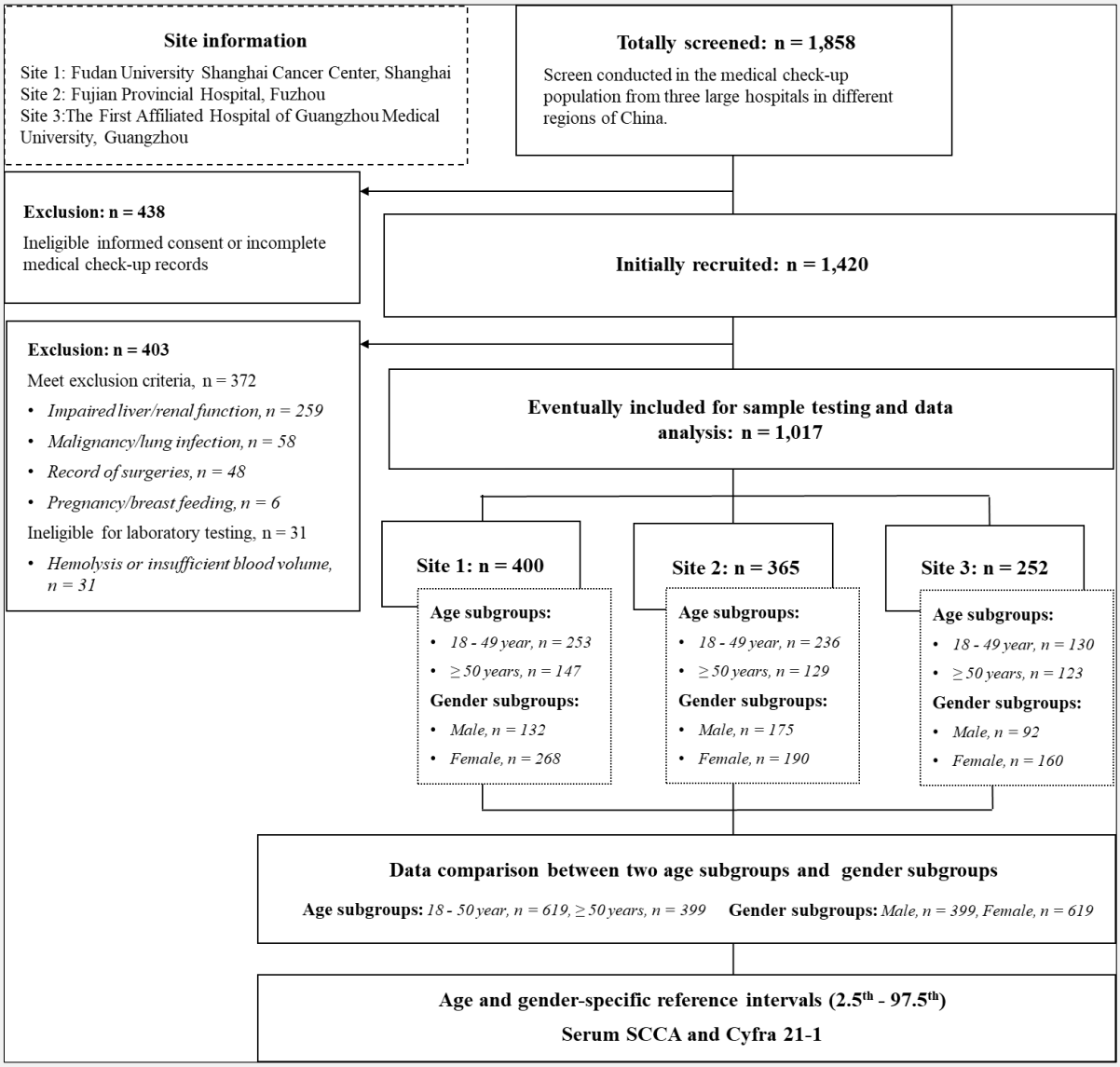

Figure 1. Study flowchart.

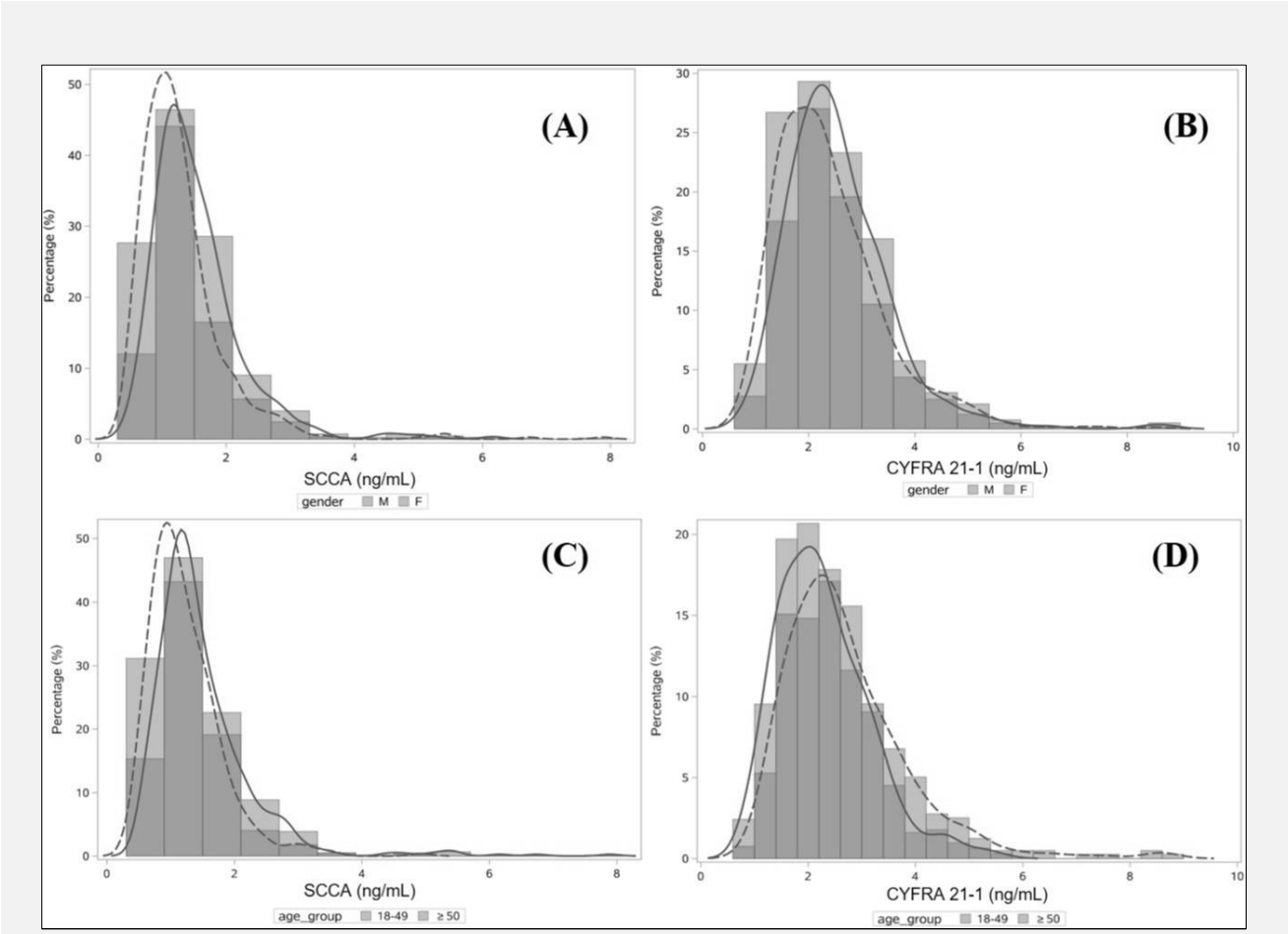

Figure 2. Distribution of serum SCCA levels in subgroups stratified by (A) gender and (B) age and serum CYFRA 21-1 levels in subgroups stratified by (C) gender and (D) age.

SCCA and CYFRA 21-1 screening using the upper limit of reference interval

As the reference intervals were established using the 0 - 95th percentile, 5% of the study population will not be included in this range, and thus the false positive rate of using the upper limit of the reference intervals for SCCA and CYFRA 21-1 for screening in this study should be 5%. When applying the 95th percentile as the upper limit for SCCA (2.7 ng/mL) and CYFRA 21-1 (3.3 ng/mL), reported on the manufacturer's instruction as the cutoff value for medical screening, the false positive rate increased from 5% to 7.96% and 16.12%, respectively (Table 4).

DISCUSSION

In this study, we established the 0 - 95th reference intervals for SCCA and CYFRA 21-1 in an apparently healthy population undergoing medical check-ups in China, and we also obtained a series of age- and gender-specific reference intervals. The 2.5th and 97.5th percentiles have also been provided in our studies, as some laboratories also tend to use 2.5 - 97.5th reference intervals for their routine works [18,22]. Moreover, this study represents the first to investigate the influence of benign nodule status on the serum level of two biomarkers in an apparently healthy population. It has been demonstrated that ethnicity can affect reference values for biochemical markers, including serum cancer biomarkers [20]. In China, clinical laboratories often use reference intervals established for foreign populations, which may not be suitable for clinical practice. Therefore, it is essential to establish reference intervals specifically for the Chinese population.

The higher values of SCCA determined in the healthy Chinese and European population have been mentioned in the manufacturer's insert. Specifically, the 95th and 97.5th percentiles (ng/mL) of the Chinese population in the manufacturer's insert are close to that reported in our

study, while higher than those of the European population [2.7 vs. 2.64 vs. 2.3; 3.0 vs. 3.0 vs. 2.7]. Except from the manufacturer's insert, only one study reported the reference range of serum SCCA levels in apparently healthy Chinese subjects [21]. In that study, 146 Chinese and 153 healthy European subjects were enrolled and set as the control group to assess the clinical performance of SCCA in differentiating cervical, lung, and head and neck cancer. The 95th and 97.5th percentiles of SCCA for apparently healthy Chinese women in this study and ours were also close in value and lower than that determined in the European cohort. Moreover, the narrower CIs determined in our study can be attributed to the larger sample size (146 vs. 1,017). Similarly, CYFRA 21-1 levels in healthy subjects are typically reported as the control group. In our study, the 95th/97.5th percentile (ng/mL) was found to be 4.39/4.93, which is higher than the values reported for the European population (manufacturer's instruction, 95th percentile: 3.3), the Korean population (97.5th percentile: 3.84 ng/mL) [22], and another Chinese population from a single-center study reported by Zhao et al. (97.5th percentile: 4.74 ng/mL) [23]. Considering the multicenter design of our study, we assume that our CYFRA 21-1 reference interval could be more representative of the apparently healthy Chinese population. Overall, our study suggests that the serum SCCA and CYFRA 21-1 levels in apparently healthy Chinese subjects may be higher than their European counterparts, highlighting the need for establishing population-specific reference intervals for these biomarkers.

Benign pulmonary and thyroid nodules are commonly observed during medical check-ups. Several studies have investigated the potential clinical value of SCCA and CYFRA 21-1 in differentiating benign and malignant lung nodule(s) in a Chinese population [24-26]. As these nodules are non-cancerous growths in the lungs or thyroid gland, they usually will not secret a significant amount of SCCA and CYFRA 21-1, leading to elevated serum levels. However, some studies have suggested that certain factors associated with the nodule(s), such as benign conditions, inflammatory skin diseases, and proliferation of tissue surrounding the nodule(s) (e.g. normal squamous cell epithelia), may slightly alter the serum levels of the two biomarkers [27-29]. These factors could potentially result in false positive results during lung cancer screening in annual medical health check-ups. Therefore, we investigated the influence of benign nodule status on serum SCCA and CYFRA 21-1 levels. No statistically significant differences between the subgroup with no benign nodule(s) and other subgroups with lung and/or thyroid nodules were determined. This suggests that the benign nodule status in apparently healthy subjects should not influence their serum SCCA and CYFRA 21-1 levels. Therefore, establishing reference intervals based on nodule status is unnecessary.

We also established a series of age- and gender-specific SCCA and CYFRA 21-1 reference intervals due to the statistically significant differences observed among age and gender subgroups. However, we acknowledge that the clinical utility of these specific reference intervals may be limited.

The positive association between age and serum CYFRA 21-1 levels observed in our study aligns with previous research [22,23,30]. Firstly, aging is associated with significant changes in the structure and function of the lungs. Age-associated dysfunction in alveolar epithelial cells may promote oxidative stress and apoptosis [31]. Therefore, CYFRA 21-1 level can be increased in older subjects as its release is associated with the death/apoptosis of epithelial cells in the aging lungs [32]. Additionally, kidney function also declines with age in healthy aging subjects [33]. Since CYFRA 21-1 is eliminated through the kidneys, this decrease in kidney function could result in higher serum CYFRA 21-1 levels due to the declining function in elimination control. However, similar studies exploring the association between age and serum SCCA levels are relatively limited. This negative association with age has also been indicated in clinical research [34] and could be explained by the decrease in cellular turnover rate with age, which impairs the ability of normal squamous cells to produce SCCA, and thus results in a negative association of SCCA level with age [35]. It is important to note that while establishing age-specific reference intervals based on these findings and existing literature is necessary, their clinical utility in screening for lung cancers may be limited. This is because the difference in serum levels of these two biomarkers between the two age subgroups of apparently healthy subjects is considerably smaller compared to the difference between cancer patients and healthy controls. For instance, in the case of CYFRA 21-1, the mean (SD) level (ng/mL) in 65 cancer patients is higher than the control group comprising 60 healthy subjects: 76.6 (10.4) vs. 7.8 (3.2), $p < 0.01$. Conversely, the difference between the two age groups in our study is only approximately 0.5 ng/ml, as 2.28 (0.86) vs. 2.71 (1.17).

Previous studies have also reported higher serum CYFRA 21-1 and SCCA levels in apparently healthy Chinese males compared to females. For CYFRA 21-1, this might be relevant to the higher prevalence of smoking in males, but the correlation between CYFRA 21-1 and smoking status remains controversial [19]. The gender difference in SCCA levels is less understood and was not significant in a previous study [21]. Similar to the age-specific reference intervals, although the gender-specific reference intervals for these two biomarkers were statistically significant, the differences in values were minimal and much lower than the differences observed between healthy individuals and those with cancer. Therefore, the gender-specific differences in CYFRA 21-1 and SCCA levels may not have strong clinical significance, suggesting that using gender-specific reference intervals in clinical practice may not be necessary. Overall, our findings suggest that the influence of benign nodule status, age, and gender on serum

SCCA and CYFRA 21-1 levels may be minimal. Therefore, the reference intervals established based on the whole population should be appropriate for clinical utilization.

It is important to note that serum levels of SCCA and CYFRA 21-1 demonstrated significant variation among the three sites (all $p < 0.05$, see Table S3). A similar variation was reported in a previous multicenter study that established reference intervals for six tumor markers for lung cancer, including SCCA and CYFRA 21-1. The distribution of the two biomarkers also varied among the 9 participating hospitals in different regions of China [34]. In our opinion, three main types of difference could contribute to this site-specific bias: 1) laboratory-related differences, such as sample collection and measurement; 2) demographic-related differences, such as gender and age, which have been observed in two other studies conducted in the Chinese population [18, 35]; and 3) clinical-related differences, such as smoking status and weight loss [40]. However, in line with our attitude toward establishing age- and gender-specific reference intervals, although there is statistical significance in the upper percentiles among the sites, their clinical significance might be limited. As shown in Table S3, the differences in the upper 95th percentile among the three sits are less than 0.29 ng/mL for SCCA and less than 0.32 ng/mL for CYFRA 21-1. Similarly, for the upper 97.5th percentile, we observed small differences of less than 0.39 ng/mL for SCCA and less than for 0.11 ng/mL CYFRA 21-1. Therefore, establishing site-specific reference intervals may not be necessary. This is because the difference in serum levels of these two biomarkers among the three sites is also considerably smaller than between cancer patients and healthy subjects.

Several biomarkers have been evaluated or are currently being evaluated as screening tests for early cancer detection, owing to the advantages of being noninvasive, radiation exposure-free, and easy to use. However, these biomarkers also have drawbacks, including low sensitivity, low specificity, and limited performance in cancers with low prevalence [37]. No biomarkers have been recognized as standalone candidates for effective early lung cancer screening. However, a recent study suggested that combining biomarker measurements with LDCT could enhance lung cancer screening strategies [4]. In China, LDCT is more affordable than in the United States, and it is widely used for lung cancer screening in medical check-ups. Measuring serum biomarkers is also common in China, although its medical value remains uncertain. This difference in approach to biomarker screening in China is likely due to the country's high burden of lung cancer, challenges in identifying high-risk patients due to the large population, and healthcare policies that support reimbursement for cancer biomarker tests. Given the clinical practices in China, it is crucial to investigate the lung cancer screening performance of serum SCCA and CYFRA 21-1.

The upper reference limit of biomarkers in a healthy reference population is commonly used as a cutoff value for disease diagnosis, and previous studies have demonstrated that CYFRA 21-1 concentrations exceeding the upper reference limit have been associated with unfavorable cancer prognosis [38,39]. In our study, we observed that the upper limits of SCCA and CYFRA 21-1 in apparently healthy Chinese subjects are higher than their European counterparts (3.00 ng/mL vs. 2.7 ng/mL and 4.93 ng/mL vs. 3.3 ng/mL). When applying the upper reference limit established for a European population to our study population, the false positive rate of SCCA and CYFRA 21-1 increased to 7.2% and 18.54%, respectively, compared to the 5% false positive rate determined through statistical analyses in our study. This finding suggests that the upper reference limit established in our study may be more suitable for lung cancer screening in Chinese medical health check-ups.

The limitations of this study should be addressed: 1) this study did not include subjects from infancy to adolescence. Since it has been reported that the concentrations of many tumor makers fluctuate with the physiological changes that occur throughout childhood [10], further studies on the serum SCCA and CYFRA 21-1 levels of these populations are necessary; 2) the controversy surrounding biomarker screening should be noted. There is a potential for overdiagnosis when using cancer biomarkers in China. In other words, although SCCA and CYFRA 21-1 have been used off-label for lung cancer screening in various regions of China due to specific national conditions, their effectiveness has not been recognized by international clinical guidelines and requires further investigation; 3) the reference intervals established in our study are only suitable for clinical laboratories using same assays and platform. 4) A previous study has suggested that current smoking and weight loss may also be considered as factors influencing serum CYFRA 21-1 levels in individuals without cancer [40]. However, due to the medical check-up setting of this study, this information was not collected, and thus cannot be evaluated by using statistical analyses. Therefore, our results on CYFRA 21-1 could be biased due to this reason.

CONCLUSION

This study established overall, age-specific and gender-specific SCCA and CYFRA 21-1 reference intervals in apparently healthy Chinese subjects. The results suggested that the influence of benign nodule status, age, and gender on the reference intervals is minimal. Altogether, this study suggests that the overall reference intervals can be used as a valuable basis for annual medical check-ups, and the upper limit of these reference intervals could be utilized as a screening tool for potential lung cancers.

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Ethics Approval and Consent to Participate:
This study is strictly in accordance with the Declaration of Helsinki and was approved by the Ethics Committees of Cancer Hospital of Fudan University (no. 050432-4-1212B), The First Affiliated Hospital of Guangzhou Medical University (No. K2018-12-029) and Fujian Provincial Hospital (No. 2019-007). All participants enrolled for sample testing and statistical analysis have signed the informed consent forms.

Data Availability Statement:
All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding authors.

Declaration of Interest:
The authors declared that they have no conflicts of interest.

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