*Original Article*

# Estimation of reference interval for neutrophil activity evaluation systems: a interim report

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**Neutrophils play an important role in innate immunity and produce reactive oxygen species, but they can also cause inflammation and oxidative stress that can damage their own tissues. We have developed neutrophil activity evaluation systems that simultaneously monitors superoxide radicals and hypochlorite ions secreted by stimulated neutrophils in a few microliters of whole blood and have conducted clinical studies in humans. Here, we report normal reference intervals with our systems based on the results of 3,082 persons who underwent comprehensive cancer screening between February 2020 and March 2022. A total of 344 were extracted as reference individuals based on the results of the cancer screening and the reference intervals of the two systems were interim estimated considering gender and age. Reference intervals can be used as a marker of sub-clinical inflammation, which is difficult to detect with other blood markers.**

***Key Words*:** **neutrophil activity, reference interval, reference individual, myeloperoxidase, reactive oxygen species**

Neutrophils play an important role in innate immunity and produce reactive oxygen species (ROS). Activated neutrophils produce the superoxide radical ($O_2^{\bullet-}$), a precursor of hydrogen peroxide ($H_2O_2$), as a primary metabolite catalyzed by NADPH oxidase. Subsequently, an enzymatic reaction of myeloperoxidase (MPO) present in neutrophil granules produces hypochlorite ions ($OCl^-$) from $H_2O_2$ and chloride ion ($Cl^-$).[1,2] Measuring $O_2^{\bullet-}$ and $OCl^-$ separately may detect subtle changes in physical status that are not expressed by changes in neutrophil counts.[3–6]

Previously, measurements of ROS and MPO activities requires separation of neutrophils from several ml of blood, which is a time-consuming process and may stress neutrophils.[7,8] It includes flow cytometry requiring extensive precautions during sample preparation to obtain reproducible results.[9] Because of the technical difficulty, neutrophil count was often used as a simpler substitute. In order to provide simpler measurements of ROS and MPO activities, we have developed a simultaneous chemiluminescence (CL) and fluorescence (FL) monitoring system. This new system allows simultaneous measurement of chemiluminescence of $O_2^{\bullet-}$ (CL-$O_2^{\bullet-}$: representing information of neutrophil quantity) and fluorescence of $OCl^-$ (FL-$OCl^-$: representing information of neutrophil quality) semi-automatically from small blood samples by using CL and FL detection reagents.[3,10] With the new system, we have reported association of FL-$OCl^-$ and clinical or sub-clinical inflammation[3,4,11] and related pathology.[12] However, they have been used as an individual monitoring tool requiring multiple measurements to investigate changes from baseline condition due to lack of normal reference intervals. As far as we know there have been no reports regarding the quantitative measurements of ROS and MPO activities in normal individuals. Settling reference intervals was technically difficult because 1. it will need strict criteria to define normal subjects because even sub-clinical level of minor inflammation may show abnormal results, and 2. data-distribution of normal subjects have not been reported previously.

The purpose of present study was to define reference intervals of quantitative measurements of $O_2^{\bullet-}$ (CL-$O_2^{\bullet-}$) and $OCl^-$ (FL-$OCl^-$) using CFL-H2200 and FLP-H3200 (Hamamatsu Photonics K.K., Hamamatsu, Japan). Reference subjects were carefully selected from a cohort of normal subjects based on a comprehensive cancer screening program using multiple medical imaging modalities including whole body [$^{18}$F] fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT) to exclude possible sub-clinical inflammation which may affect neutrophil activities. Obtained normal values were investigated for the distribution to select proper transform function to define reference intervals.

## Materials and Methods

**Study design.** This is a cross-sectional study to establish reference intervals for neutrophil activity evaluation systems by selecting reference individuals. Reference individuals were selected based on certain exclusion criteria based on information from medical interviews, blood tests, PET and CT examinations.[13]

**Study participants.** The study included 3,082 volunteers who underwent the cancer screening program with multiple medical imaging modalities at the Hamamatsu Medical Imaging Center from February 2020 to March 2022. All participants provided written informed consent. This study was conducted in compliance with the Declaration of Helsinki and was approved by the Ethics Committee of the Hamamatsu Medical Photonics Foundation (study number B024). In case a subject underwent multiple check-up examinations during the inclusion period, only first data was used for the analyses. As a result, a total of 2,677 volunteers were included for the following investigations.

**Selection of reference individuals.** In order to select normal subjects for a reference population, subjects with positive ongoing pathology were excluded based on the past medical history and the results of the imaging examinations. The medical

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**Table 1.** Characteristics of the Participants

| Charasteristic | Unit | Reference popuration *n* = 3,082 | Reference individual *n* = 344 |
|---|---|---|---|
| Sex | M | 1,963 | 201 |
| | F | 1,119 | 143 |
| Age | years | 58.00 ± 10.67 | 49.17 ± 7.43 |
| BMI | kg/m$^2$ | 23.37 ± 3.50 | 21.45 ± 2.49 |
| Systoric blood presure | mmHg | 124.17 ± 17.27 | 115.31 ± 14.00 |
| Diastoric blood presure | mmHg | 73.91 ± 17.27 | 69.33 ± 10.50 |
| HbA1c (NGSP) | % | 5.80 ± 0.59 | 5.52 ± 0.26 |
| Neutrophil | % | 57.80 ± 8.47 | 58.10 ± 8.36 |
| Lymphocyte | % | 33.59 ± 7.83 | 33.49 ± 7.47 |
| COVID-19 vaccine | Unvaccinated | 2,159 (70%) | 245 (71%) |
| | Vaccinated | 923 (30%) | 99 (29%) |

Continuous variables are expressed as means ± SD. Test methods; HbA1c (latex agglutination method), neutrophil and lymphocyte (flow cytometry).

**Table 2.** Features of the device for neutrophil activity evaluation system

| System | Key reference | Measurement | Detector | Channels | Size (mm) | Weight (kg) |
|---|---|---|---|---|---|---|
| CFL-H2200 | Ref (10) | CL and FL | PMT | 2ch | W123 × D232 × H204 | 3.4 |
| FLP-H3200 | This study | FL only | PD | 2ch | W81 × D85 × H149 | 0.9 |

PMT, photomultiplier tube; PD, Si photodiode.

imaging for the cancer screening program consisted of whole body FDG PET/CT, brain and pelvic MRI, and ultrasound of abdomen and breast (females only). First, 1,316 subjects were excluded from the reference population due to the following past medical history; malignancy, hypertension, diabetes, dyslipidemia, hyperuricemia, heart disease, anemia, respiratory disease, digestive disease, urologic disease, brain disease, thyroid disease, and those who reported taking sleeping pills, tranquilizers or painkillers. For the remaining 1,361 participants, results of a series of imaging examinations were investigated for the positive ongoing pathologic findings. Since this study was designed to estimate reference intervals for the inflammatory markers, particular attention was paid to exclude possible subclinical inflammatory process. For example, inflammatory FDG uptake on whole body PET/CT, or vascular calcification on x-ray CT was considered as a specific marker of inflammation and excluded from the reference population. For 70% of the subjects who have been vaccinated for COVID-19, cancer screening was done at least 4 weeks after the recent vaccination. Since chronic inflammatory effect of COVID-19 vaccination was unknown, they were included for the reference population and investigated for the possible subclinical inflammatory effects. After excluding 1,017 subjects with positive imaging results, the final study population consisted of 344 healthy normal subjects without any history of ongoing pathology or inflammation (Table 1).

**Measurement of neutrophil activity.** In this study, two types of systems were used to evaluate neutrophil activity, CFL-H2200[3] and the newly developed FLP-H3200, which is a prototype of only $OCl^-$ measurement (Table 2). FL-$OCl^-$ measured with CFL-H2200 was defined as FL-$OCl^-$_CFL, and FL-$OCl^-$ measured with FLP-H3200 was defined as FL-$OCl^-$_FLP. Both systems use the same fluidic-chip[3] optimized for blood measurement as the sample container. These systems are connected to a PC via a USB cable for control and measurement. Participants were fasted for at least 4 h and blood was drawn from their forearms for biochemical testing. About 200 µl of blood for neutrophil activity evaluation was divided into anticoagulant container BD Microtainer (Becton Dickinson and Company, Franklin Lakes, NJ) and was used to measurement within 2 h after blood collection. Three µl of whole blood was incubated in a reaction mixture containing specific chemiluminescent reagent 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo [1,2-a] pyrazin-3-one hydrochloride (MCLA; Tokyo Kasei, Tokyo, Japan) and fluorescent reagent aminophenyl fluorescein (APF; GORYO Chemical, Sapporo, Japan). Subsequently CL-$O_2^{\bullet-}$ and FL-$OCl^-$ produced by phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Japan, Tokyo, Japan) stimulation were simultaneously monitored for 1,500 s. The details are previously described.[3,4] The fluorescence and chemiluminescence signal increased by PMA stimulation were calculated by a dedicated analysis software and used as evaluation values. The above operations were performed without disclosing the sample information in order to avoid the bias of the operator.

**Estimation of reference intervals.** The reference intervals of neutrophil activity were estimated according to the Tango's method.[14–16] First, distribution of measured activities was investigated for the possible inflammatory effects of COVID-19 vaccination. In case the effect was positive, vaccinated participants were excluded from the further analyses. Second, sex-related difference was investigated to determine the need for separate reference intervals for males and females. Third, the distribution of measured activities was transformed into the Gaussian form using one of the following transformation functions based on the Akaike information criterion (AIC);[17]

(a) X
(b) LogX
(c) $X^{\lambda}$

The reference intervals of each activity were defined as between the 2.5 and 97.5 percentiles of the transformed data distribution from the selected reference population. For the CL-$O_2^{\bullet-}$, transformed reference values were plotted against participants' age to visualize age-dependent reference values. Age-dependent reference values were not investigated for the FL-$OCl^-$, because the sample size became too small to estimate after stratification by gender and age.


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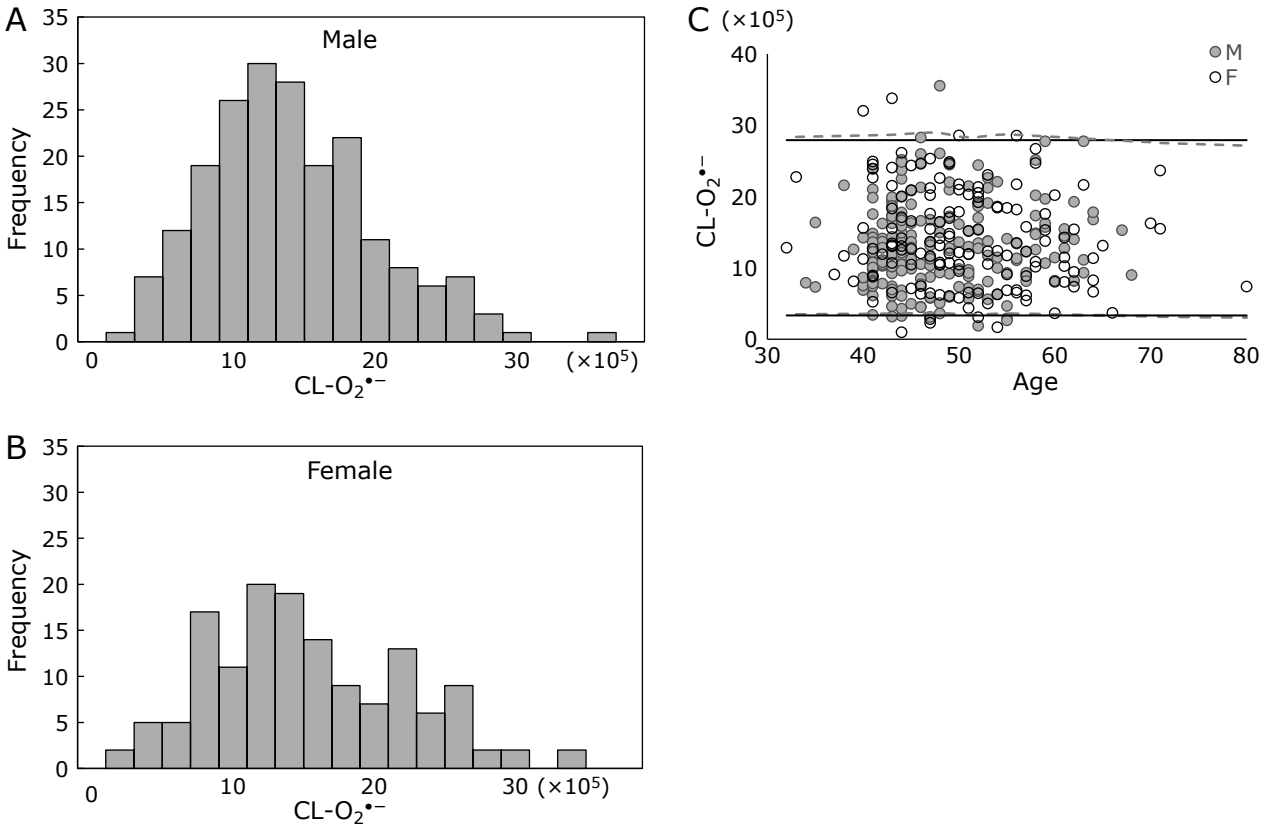


**Fig. 1.** CL graphs of reference individuals. (A) The histogram of males CL-$O_2^{\bullet-}$. (B) The histogram of females CL-$O_2^{\bullet-}$. (C) Age distribution of males and females. The CL-$O_2^{\bullet-}$ of reference individuals were plotted by age. The solid lines show the estimated upper and lower limits of the reference intervals for all individuals, and the dotted lines show the estimated values for each age.

**Statistical analysis.** The effects of COVID-19 vaccination and gender were determined using Welch Modified Two-sample *t* test and Wilcoxon rank-sum test. The correlation between systems was performed using Spearman's rank correlation coefficient. These analyses were performed using SPSS ver. 26 (IBM SPSS Statistics, Chicago, IL). The statistically significance level of the test was considered two-tailed $p<0.05$.

## Results

**Estimating the reference interval of CL-$O_2^{\bullet-}$.** The effect of COVID-19 vaccination was negative with Welch Modified Two-sample *t* test ($p = 0.6773$) or Wilcoxon rank-sum test ($p = 0.4552$). Figure 1 shows distribution of CL-$O_2^{\bullet-}$ of males (A), and females (B). There was no significant difference in the CL-$O_2^{\bullet-}$ measurements of 344 reference individuals between males and females either with Welch Modified Two-sample *t* test ($p = 0.1412$) or Wilcoxon rank-sum test ($p = 0.1898$). AIC and other transformation parameters were summarized in Table 3 for the total of 344 reference individuals. Among the tree models (a, b, and c), the best transform function was (c) $X^{\lambda}$ with AIC = 471.15 and $\lambda = 0.53$. Using this transform function, the 2.5 and 97.5 percentiles was the CL-$O_2^{\bullet-}$ = 3.33 ($\times 10^5$) and 27.90 ($\times 10^5$), respectively (Table 4 and Fig. 1C). The age-specific reference intervals are shown by the dotted lines (Fig. 1C). No characteristic changes with age were observed visually or numerically.

**Estimating the reference interval of FL-$OCl^-$_CFL.** The effect of COVID-19 vaccination was statistically significant difference with Welch Modified Two-sample *t* test ($p<0.001$) or Wilcoxon rank-sum test ($p<0.001$). Therefore, only 245 unvaccinated individuals were used. Figure 2A shows distribution of FL-$OCl^-$_CFL of male ($n = 135$, above), and female ($n = 110$, below). There was statistically significant difference in the FL-$OCl^-$_CFL measurements of reference individuals between males and females either with Welch Modified Two-sample *t* test ($p = 0.0111$) or Wilcoxon rank-sum test ($p = 0.0113$). For male, the best transform function was (b) LogX with AIC = −20.68 (Table 3). Using this transform function, the 2.5 and 97.5 percentiles was FL-$OCl^-$_CFL = 1.49 ($\times 10^3$) and 6.29 ($\times 10^3$), respectively (Table 4 and Fig. 2B). For female, the best transform function was (b) LogX with AIC = 10.70 (Table 3). Using the transform function, the 2.5 and 97.5 percentiles was FL-$OCl^-$_CFL = 1.25 ($\times 10^3$) and 5.73 ($\times 10^3$), respectively (Table 4 and Fig. 2B). Because of stratification by gender and age, the sample size became small and the precision of the estimates for each age was poor and could not be calculated.

**Table 3.** Optimal transformation parameters

| Item | Sex | Type | *k* | *m* | λ | AIC |
|---|---|---|---|---|---|---|
| CL-$O_2^{\bullet-}$ | Total | $X^{\lambda}$ | 0 | 0 | 0.5 | 471.2 |
| FL-$OCl^-$_CFL | M | LogX | 0 | 0 | | −20.68 |
| | F | LogX | 0 | 0 | | 10.7 |
| FL-$OCl^-$_FLP | M | LogX | 0 | 0 | | 127.6 |
| | F | LogX | 0 | 0 | | 135.9 |

*k*, number of lower outliers; *m*, number of upper outlieres; λ, parameter value of coresponding power transformation; AIC, Akaike information criterion.

**Estimating the reference interval of FL-$OCl^-$_FLP.** The effect of COVID-19 vaccination was statistically significant

**Table 4.** Means and reference intervals of neutrophil activity evaluation system for each device

| Item | Unit | Sex | Reference population | | Reference individual | | Reference intereval | |
|---|---|---|---|---|---|---|---|---|
| | | | *n* | Mean ± SD | *n* | Mean ± SD | Lower limit | Upper limit |
| CL-$O_2^{\bullet-}$ | $\times10^5$ | M + F | 3,082 | 14.76 ± 6.77 | 344 | 13.55 ± 6.33 | 3.33 | 27.90 |
| FL-$OCl^-$_CFL | $\times10^3$ | M + F | 3,082 | 3.55 ± 1.50 | 245 | 3.10 ± 1.23 | — | — |
| | | M | — | — | 135 | 3.28 ± 1.29 | 1.49 | 6.29 |
| | | F | — | — | 110 | 2.88 ± 1.13 | 1.25 | 5.73 |
| FL-$OCl^-$_FLP | $\times10^3$ | M + F | 3,082 | 5.72 ± 2.48 | 245 | 4.94 ± 2.11 | — | — |
| | | M | — | — | 135 | 5.20 ± 2.19 | 2.16 | 10.63 |
| | | F | — | — | 110 | 4.61 ± 1.97 | 1.79 | 9.90 |

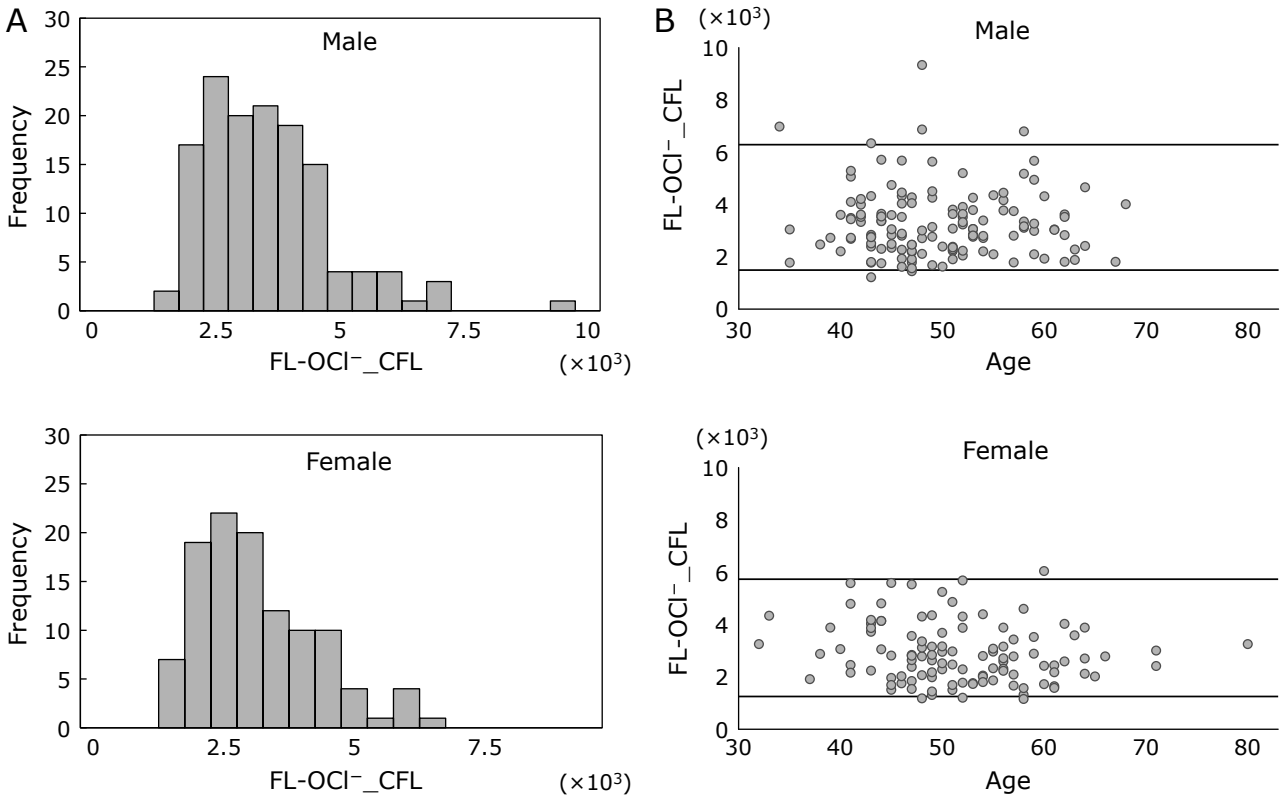


**Fig. 2.** FL-$OCl^-$_CFL graphs of reference individuals. The upper graphs show males, and the lower graphs show females. (A) The histograms of FL-$OCl^-$_CFL. (B) Age distributions of FL-$OCl^-$_CFL. The FL-$OCl^-$_CFL of reference individuals were plotted by age. The solid lines show the estimated upper and lower limits of the reference interval for all individuals.

difference with Welch Modified Two-sample *t* test ($p<0.001$) or Wilcoxon rank-sum test ($p<0.001$). Therefore, only 245 unvaccinated individuals were used similar to FL-$OCl^-$_CFL. Figure 3A shows distribution of FL-$OCl^-$_FLP of male ($n = 135$, above), and female ($n = 110$, below). There was a statistically significant difference in the FL-$OCl^-$_FLP measurements of reference individuals between males and females either with Welch Modified Two-sample *t* test ($p = 0.0275$) or Wilcoxon rank-sum test ($p = 0.0332$). For male, the best transform function was (b) LogX with AIC = 127.61 (Table 3). Using this transform function, the 2.5 and 97.5 percentiles was FL-$OCl^-$_FLP = 2.16 ($\times10^3$) and 10.63 ($\times10^3$), respectively (Table 4 and Fig. 3B). For female, the best transform function was (b) LogX with AIC = 135.87 (Table 3). Using this transform function, the 2.5 and 97.5 percentiles was FL-$OCl^-$_FLP = 1.79 ($\times10^3$) and 9.90 ($\times10^3$), respectively (Table 4 and Fig. 3B). The estimates for each age could not be calculated for the same reason as FL-$OCl^-$_CFL. In order to evaluate the performance of the newly developed system, we investigated the correlation with the conventional system. The correlation coefficient between FL-$OCl^-$_CFL and FL-$OCl^-$_FLP was 0.961 (Fig. 4).

## Discussion

This study estimated reference intervals for neutrophil activation parameters using the Tango's method for normalizing distribution. The reference interval for the CL-$O_2^{\bullet-}$ was not affected by gender, while those of FL-$OCl^-$ showed different distribution, which requires separated reference intervals for males and females. Effect of prior COVID-19 vaccination was negative for CL-$O_2^{\bullet-}$, but positive for FL-$OCl^-$.

Neutrophils play essential role in ROS-dependent antimicrobial actions. In practice, neutrophil ROS measurement is a key to diagnose leukocyte dysfunction such as chronic granulo-


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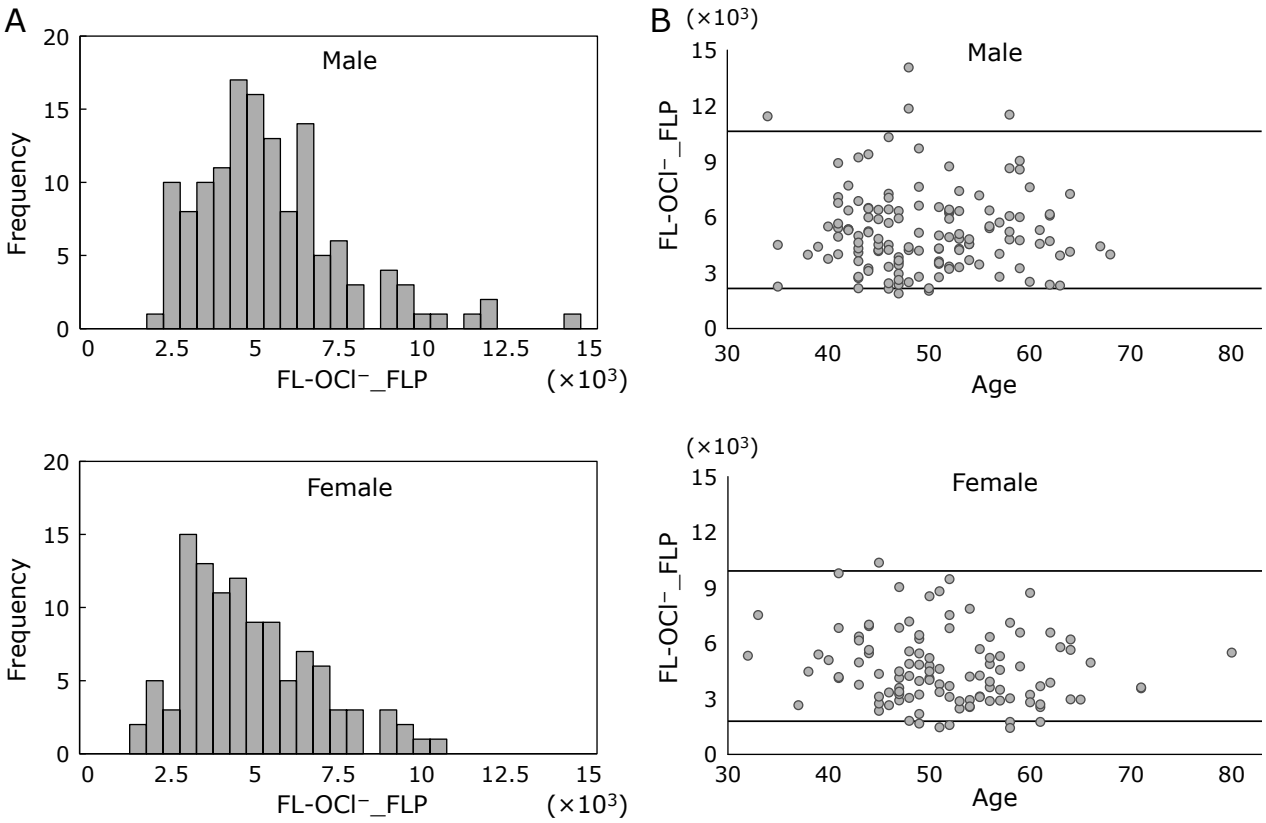


**Fig. 3.** FL-OCl⁻_FLP graphs of reference individuals. The upper graphs show males, and the lower graphs show females. (A) The histograms of FL-OCl⁻_FLP. (B) Age distributions of FL-OCl⁻_FLP. The FL-OCl⁻_FLP of reference individuals were plotted by age. The solid lines show the estimated upper and lower limits of the reference interval for all individuals.

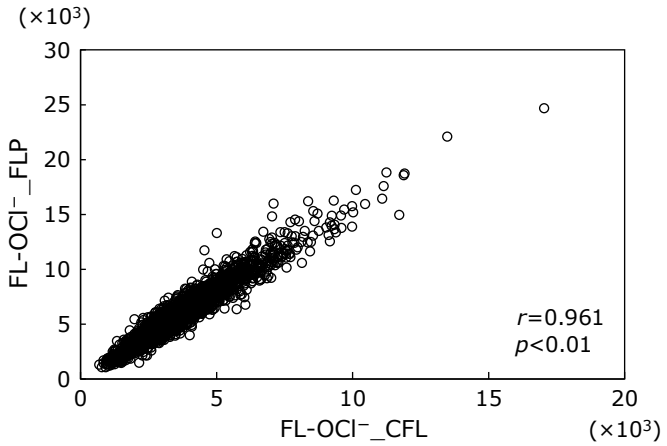


**Fig. 4.** Comparison of neutrophil activity evaluation systems. Correlation between FL-OCl⁻_CFL and FL-OCl⁻_FLP.

matous disease (CGD).[18] However, excessive increase in ROS levels has harmful effects on cell homeostasis, structures, and functions and results in oxidative stress.[19] Our previous investigations showed that $O_2^{\bullet-}$ closely related to neutrophil count, while $OCl^-$ related to neutrophil quality, that is, the ability to produce ROS.[3] FL-$OCl^-$ has been found to be associated with inflammation and reflects more subtle changes in bodily condition than CL-$O_2^{\bullet-}$.[3,4,11]

In the present study, the reference interval for CL-$O_2^{\bullet-}$ was not affected by gender, while those of FL-$OCl^-$ showed different distribution, which requires separated reference intervals for males and females. Despite our expectation, the reference interval of CL-$O_2^{\bullet-}$ did not show gender- or age-dependency. Gender- and age-related differences in neutrophil counts and neutrophil activity have been reported,[20,21] which differ from our results. The present study population consisted of highly selected normal subjects from a cancer screening program, thus may limit the variation to demonstrate gender-dependent changes. In addition, lack of variation in age (40–65 years) in the present study population may limit the demonstration of age-dependent trends. $O_2^{\bullet-}$ production is known to depend on neutrophil counts, which did not show gender or age-dependency after 18 years old.[22] Regarding FL-$OCl^-$, a significant difference was observed between males and females (Fig. 2 and 3). Difference between estrogen and testosterone in immunological role may explain the gender differences in FL-$OCl^-$.[22] The reference interval of female showed a trend toward higher values for those under 45 years of age. The results are in line with higher neutrophil count during puberty and adulthood in female.[22]

The present study collected reference individuals from cancer screening program including advanced medical imaging consisting of whole body FDG PET/CT, pelvic and brain MRI and abdominal ultrasound. It is one of the most detailed health check-up programs for early diagnosis of not only cancer, but also other non-malignant adult disorders. Reference population in the present study are considered truly normal in terms of inflammatory oxidative stress, because FDG PET/CT is particularly useful to demonstrate chronic inflammation,[23] which lies under most pre-symptomatic adult diseases with excessive oxidative stress.[24]

This study employed a new procedure for estimating reference intervals, which is optimal in having a good balance with the number of independent parameters included.[16] Classically, the reference interval of clinical laboratory tests has been defined as that between the 2.5 and 97.5 percentiles of the data distribution in either Gaussian or a log-Gaussian distribution from a healthy population.[16] When the observed data follow neither a Gaussian nor a log-Gaussian distribution, they have used non-parametric percentile estimates.[16] As a result, the classical method for reference interval estimation frequently results in high degree of imprecision and is sensitive to the presence of outliers due to non-parametric estimates.[16] Converting a non-Gaussian to a Gaussian distribution allows the use of parametric methods providing more reliable estimates of reference intervals than non-parametric methods for a given sample size. However, this conversion includes technical problem to treat outliers. The present study employed Tango's method, which solves the problem by assuming each of the appropriately transformed outliers also follows a parametric distribution.[16] This method requires additional computation process to select optimal transformation function but allows the use of parametric methods providing more reliable estimates of reference intervals than non-parametric methods for a given sample size.[16]

The present study investigated FL-$OCl^-$ using 2 different systems. The measurements of the two systems (FLP-H3200 and CFL-H2200) showed a good correlation ($r = 0.961$), without a trend of over or underestimation (Fig. 4). We developed the new popularization model (FLP-H3200) for the healthcare market as an inexpensive and convenient tool to measure only $OCl^-$. In contrast, CFL-H2200 is an original system developed as a model for researchers that can simultaneously monitor the primary metabolite produced by neutrophils $O_2^{\bullet-}$ and the secondary metabolite $OCl^-$ over time (Table 2). The new system focused $OCl^-$ because cellular ROS production capacities are well contained in the $OCl^-$ signal, a secondary metabolite, rather than the $O_2^{\bullet-}$.[3] Limiting measurements of only $OCl^-$ simplified the optical configuration and made it possible to set the excitation light intensity high enough to obtain a sufficient amount of signal. Thus, the detector was able to be substituted to inexpensive on-board Si photodiode (PD) from the expensive and highly sensitive photomultiplier tube (PMT). The system is otherwise identical in terms of sample preparation and measurement process.[3] The present results demonstrated the new FLP-H3200 has sufficient performance compared to the original high-precision model CFL-H2200.

The present study successfully defined reference intervals for CL-$O_2^{\bullet-}$ and FL-$OCl^-$. Despite their clinical importance, they have been used as a monitoring tool because normal reference intervals have not been defined. Introduction of reference interval allows measurement of neutrophil activities through a single examination. Measurements with inexpensive FLP-H3200 will allow repeated measurements in the healthcare field.

Finally, there are several limitations to be discussed. First, whole-body cancer screening including FDG-PET is expensive, of limited availability, and not yet suitable for general use.[25,26] Higher cost of the cancer screening program may imply selection bias from relatively wealthy elderly (average age 58 years) subjects with healthy lifestyle. Second, numbers of reference population may not be enough. In the present study, only 344 reference population was selected from 3,082 participants. Strict criteria using cancer screening program will help to select true-normal subjects, but it may reduce the number of reference population. Further study with larger study population will adjust the reference intervals in future. Third, possible sub-clinical chronic inflammation of COVID-19 vaccination may affect the reference intervals in future. Although 70% of subjects in this study were unvaccinated, the proportion of vaccinated subjects will increase in the future which may affect FL-$OCl^-$.

In conclusion, reference intervals of CL-$O_2^{\bullet-}$ with CFL-H2200, and FL-$OCl^-$ with CFL-H2200 and FLP-H3200 were defined based on the highly selected normal population. The reference interval for the CL-$O_2^{\bullet-}$ was not affected by gender, while those of FL-$OCl^-$ showed different distribution, which requires separated reference intervals for males and females. This is an interim report, and further study with larger study population will adjust the reference intervals in future.

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## Conflict of Interest

TS, TM, and KK are employees of Hamamatsu Photonics K.K. YH and CI were employees of Hamamatsu Photonics K.K. when this research was conducted. KK submitted patent applications on the technologies used in this study. The other authors declare no potential conflicts of interest.

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