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SEED Haematology



The art of defining reference intervals

The importance of reference intervals

To interpret the result of a blood count, a healthcare professional needs comparative values from healthy people, so-called reference ranges or reference intervals, alongside the results of the analysis. While previous results can be helpful for the interpretation of laboratory results of existing patients, reference intervals are essential especially for the interpretation of measured values of new patients.

Determining a reference interval means defining an upper reference limit (UL) and a lower reference limit (LL). Both limit values are based on the values measured and statistically analysed within the reference population for the parameters.

An appropriate reference population has to represent the biological variability among healthy individuals on the one hand, but also allow for the identification of pathologic samples on the other hand. In the study of L van Pelt J et al. [1], an outstandingly well characterised reference cohort was the basis for reference intervals for all 105 XN parameters.

This article will explain the statistical approach used by L van Pelt J et al. and share the reference intervals for all 105 parameters from the XN-Series analyser in a Dutch cohort.

Reference intervals vs. decision limits

A patient result outside the reference interval does not necessarily mean that medical steps are required. It represents the fact that the result does not fit with the majority of the reference population. Decision limits help the healthcare professional differentiate between a deviating value and a pathological result, which sometimes even can be within a reference interval.

More about decision limits and their differentiation to reference intervals is explained in 'SEED Reference intervals and what Sysmex can offer' [2].

Defining the reference population

To determine reference intervals for all 105 diagnostic and research parameters of an XN-Series analyser, the reference population was defined as a subgroup of the Lifelines cohort. Lifelines is a multi-disciplinary prospective population-based cohort study [3].

Lifelines

In Lifelines, three generations will be followed-up for at least 30 years, and data from questionnaires, physical examinations and biological samples is collected. The aim of Lifelines is to be a resource for the national and international scientific community. Since 2006, 167,729 persons from the North of the Netherlands have been included. Blood analyses are done with state-of-the-art XN-Series haematology analysers. Find out more details on the *Lifelines webpage* [3].

The challenge with population-based reference interval studies is how to define a 'healthy' reference cohort. Most reference interval studies use extensive self-report questionnaires, including specific laboratory tests such as HbA_{1c} and eGFR, to exclude individuals who are not healthy. This initial exclusion is often referred to as 'primary exclusion'. In most studies, further reference intervals are calculated after this initial exclusion.

In order to determine the XN reference intervals, apparently healthy individuals, participants from the Lifelines cohort, were included. Individuals were excluded if they fulfilled one or more of the following exclusion criteria:

- History of stroke
- Diabetes mellitus (self-reported; HbA_{1c} ≥ 47.5 mmol/mol; or fasting plasma glucose ≥ 7.0 mmol/L)
- Chronic liver disease
- Chronic kidney disease (self-reported; eGFR (CKD-Epi) < 60 mL/min/1.73 m²)
- Renal failure
- Pregnant women

Overall, the cohort resulted in 15,803 apparently healthy individuals from January 2014 until January 2015, aged 20–92 years, to determine the reference intervals.

Common conditions with a long latency, such as anaemia, pose a challenge to reference cohorts. Someone may still have a normal haemoglobin level but already develop some degree of microcytosis and hypochromia. In order to remove these individuals from the final cohort, a 'secondary exclusion' is recommended. In accordance with the Committee on Reference Intervals and Decision Limits (C-RIDL) of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [4, 5], the Latent Abnormal Value Exclusion (LAVE) approach to determining the reference population was followed.

The aim of LAVE is to refine the reference intervals by excluding those subjects with 'abnormal values' that are associated with chronic or latent conditions but which are not (yet) known to the people affected and therefore cannot be detected with the help of questionnaires. To identify an individual within the cohort that presents with 'abnormal values', several index parameters were defined. The authors based the selection of the index parameters on current scientific literature and selected parameters that are deemed to be associated with latent clinical conditions, such as anaemia and (chronic) inflammation.

For the reference interval calculation of the XN-Series, the following index parameters were used by the authors:

- Haemoglobin (HGB)
- Mean corpuscular volume (MCV)
- Red blood cells (RBC)
- Reticulocyte count (RET#)
- Neutrophil count (NEUT#)
- Lymphocyte count (LYMPH#)
- Monocyte count (MONO#)
- Platelets (PLT)
- Mean platelet volume (MPV)

When determining the reference interval using the example of the white blood cell count (WBC/ μ L) parameter, it means that first, all individual WBC values are included. Next, the measured values from persons with abnormal index parameter(s) are excluded. LAVE levels are defined depending on how many abnormal index parameters are accepted. LAVE abnormal O (written as LAVE(+)AbnO) means no acceptance of results outside the calculated reference intervals for all index parameters. LAVE(+)Abn1 accepts one abnormal index parameter result and LAVE(+)Abn2 accepts two results. LAVE(-) does not apply the LAVE algorithm to the dataset.

A common effect of conditions like latent anaemia or chronic inflammatory diseases is an increase of the WBC count [6]. As a consequence, in LAVE-processed reference data, the WBC count is a common gatekeeper by lowering the upper WBC reference interval limit and thus excluding individuals from the reference cohort (see Fig. 1).



Fig. 1 Simplified example for the LAVE approach on the WBC parameter The initial measured population values lead to a reference interval for WBC of between 4 and 10. Excluding those individuals with at least one abnormal index parameter (blue body) lowers the upper limit of the resulting reference interval from 10 to 9 in this example.

Reference interval calculation

The statistical approach to define the reference interval for a parameter depends on the type of data. Some parameters present a normal or so-called 'Gaussian distribution', while other parameters present a 'shifted' or 'skewed distribution'. For most skewed parameters, a conversion into a Gaussian distribution was possible by a modified Box-Cox transformation [7].

For Gaussian-distributed parameters (initially and after conversion), the lower or upper limit of a reference interval was parametrically calculated by determining the mean \pm 1.96 of the standard deviation.

For non-convertible skewed distributed parameters, the reference intervals were calculated non-parametrically by using the 2.5% and 97.5% percentiles to define the upper and lower reference interval limits.



Left: A Gaussian (normal) distribution. For calculating the reference interval, the \pm 1.96 standard deviation (SD \leftarrow) is calculated from the mean.

Right: A shifted (skewed) distribution. For calculating the reference interval, the lower and upper 2.5% percentile of the data were cut to define the reference interval.

Effects of LAVE levels on the resulting reference intervals

The stricter the LAVE algorithm is set, the narrower the resulting reference intervals are (see Fig. 2). The narrower the reference interval, the more likely a patient's values come to lay outside of the reference interval. A good balance for diagnostic purposes is given at LAVE(+)Abn1 according to C-RIDL recommendations (red marking in Fig. 2).



Fig. 2 Graphical comparison of reference interval widths for WBC to illustrate the effect of LAVE on reference intervals

LAVE(-): reference intervals derived without applying LAVE. **LAVE(+)Abn2:** two abnormal values among index parameters allowed for reference interval derivation.

LAVE(+)Abn1: one abnormal value among index parameters allowed for reference interval derivation.

LAVE(+)Abn0: no abnormal values among index parameters allowed for reference interval derivation.

The blue bar represents the 95% confidence interval. The number in the bar indicates the numerical value of the reference interval width. The red marking indicates the LAVE level that should be used for determining reference intervals recommended by the C-RIDL.

Sources of variation and the need for stratification

The term stratification in the context of determining reference intervals means splitting the reference population into subgroups and defining specific reference intervals for each subgroup.

For HGB, for example, a difference in the mean values of males and females is commonly known. To objectively assess the need for stratification with a mathematical method, the magnitude (size) of variation between subgroups of reference intervals was estimated as a standard deviation ratio (SDR) using **An**alysis **o**f **Va**riance (ANOVA).

The ANOVA test can tell whether the mean values of several independent groups differ significantly from each other just by chance or because of systematic reasons. The standard deviation ratio (SDR) is given using the ratio between the standard deviations of two subgroups (e.g., males vs. females). The need for stratification is to be considered by a specific threshold value. Consistent with current literature, the authors used a threshold of 0.4 [4, 8]. Thus, an SDR of greater than or equal to 0.4 indicates that stratification is required.

The SDR_{age} was below the threshold for all parameters. For some parameters, the SDR_{sex} exceeded the threshold. Therefore, in LAVE(+)Abn1, individual reference intervals for males and females were assessed for RBC, RBC-O, RET-RBC-Z, RPI, HGB, HGB-O, HCT and MCHC.

Verification of reference intervals into routine

Reference intervals published in a scientific journal or textbooks cannot be transferred into routine use without verification measures. The SEED article <u>'SEED Reference intervals – and what</u> <u>Sysmex can offer'</u> [2] provides more details about how the IFCC suggests validating and implementing new reference intervals in the diagnostic laboratory.

References

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- [4] Ichihara K et al. (2010): An appraisal of statistical procedures used in derivation of reference intervals. <u>Clin Chem Lab Med 48: 1537–51</u>.
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Reference intervals for all XN-Series parameters for adults according to L van Pelt J et al.

In the main manuscript of L van Pelt J *et al.* 2022, only 95 of the 105 XN-Series parameters are included due to the journal requirements related to units. In Table 1, all 105 diagnostic and research parameters based on LAVE(+)Abn1 are provided. Table 2 explains the abbreviations of the parameters.

Reference intervals for all 105 diagnostic and research parameters on XN-Series analysers

Extract from supplementary material no 2 of L van Pelt J et al.

The demonstrated medians (Me), lower (LL) and upper limits (UL) are calculated with LAVE(+)Abn1 (a single result outside the RIs allowed). The parameters are clustered in the (A) red blood cell related parameters, (B) white blood cell related parameters and (C) platelet related parameters. (A) If $SDR_{sex} \ge 0.4$ the results are given for males and females separately. (B) and (C) SDR_{sex} is less than 0.4 for all parameters, thus results are given for males and females together. Bold letters indicate diagnostic parameters, normal letters indicate research parameters. The (\$) indicates parameters that do not have a normal distribution even after log transformation. NEUT-GI corresponds to the formerly used term NE-SSC, NEUT-RI to NE-SFL, and NE-Z to NE-FSC. The (*) indicates parameters whose availability depends on the respective configuration of the XN-Series analyser.

Parameters	Unit	Mal	es and fema	es	Males			Females		
		LL	Me	UL	LL	Me	UL	LL	Me	UL
RBC	10 ¹² /L				4.4	5.1	5.7	4.0	4.5	5.2
HGB	g/L				134	152	170	118	136	152
HGB	g/dL				13.4	15.2	17.0	11.8	13.6	15.2
HGB	mmol/L				8.3	9.5	10.5	7.3	8.4	9.5
нст	L/L				0.41	0.45	0.50	0.37	0.41	0.46
MCV	fL	82.5	90.3	97.4						
MCH ^{\$}	pg	26.8	30.0	32.6						
MCH ^{\$}	amol	1662	1862	2024						
МСНС	g/L				317	336	352	311	330	346
мснс	g/dL				31.7	33.6	35.2	31.1	33.0	34.6
МСНС	mmol/L				19.7	20.9	21.9	19.3	20.5	21.5
RDW-SD	fL	37.9	42.5	48.3						
RDW-CV	%	11.8	12.8	14.3						
RET *	10 ⁹ /L	32.8	57.8	97.7						
RET *	%	0.7	1.2	2.0						
HFR * ^{\$}	%	0.00	0.60	2.33						
MFR *	%	2.5	6.3	11.9						
LFR *	%	86.2	93.1	97.6						
IRF *	%	2.7	6.9	13.8						
IRF-Y	ch	16.8	18.1	18.9						
NRBC ^{\$}	10 ⁹ /L	0.00	0.00	0.01						
NRBC ^{\$}	%	0.0	0.0	0.2						
RET-He * ^{\$}	pg	29.3	32.8	35.4						
RET-He * ^{\$}	amol	1817	1986	2195						
RBC-He * ^{\$}	pg	27.2	30.2	32.5						
RBC-He * ^{\$}	amol	1688	1875	2017						
DELTA-He * ^{\$}	pg	1.2	2.6	3.6						
DELTA-He * ^{\$}	amol	77	161	223						
DELTA-HGB	g/L	-7	0	6						
DELTA-HGB	g/dL	-0.7	0.0	0.6						
DELTA-HGB	mmol/L	-0.04	0.00	0.04						
MicroR	%	0.3	1.1	3.3						
MacroR	%	3.1	3.6	4.5						
HYPO-He * ^{\$}	%	0.0	0.1	0.4						
HYPER-He * ^{\$}	%	0.4	0.6	0.8						

Table 1 (A) – Red blood cell related parameters

% - percentage of a cell population, ch - channel, FI - fluorescence intensity, SI - scatter intensity

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Table 1 (A) – Red blood cell related parameters

Parameters	Unit	Males and females			Males			Females		
	_	LL	Me	UL	LL	Ме	UL	LL	Me	UL
RBC-O	10 ¹² /L				4.4	5.0	5.7	4.0	4.5	5.1
HGB-O	g/L				134	152	170	119	136	153
HGB-O	g/dL				13.4	15.2	17.0	11.9	13.6	15.3
HGB-O	mmol/L				8.4	9.5	10.5	7.4	8.4	9.5
MCHC-O	g/L	312	333	352						
MCHC-O	g/dL	31.2	33.3	35.2						
MCHC-O	mmol/L	19.4	20.7	21.9						
FRC ^{\$}	10 ¹² /L	0.0000	0.0000	0.0029						
FRC ^{\$}	%	0.00	0.00	0.06						
RPI					0.7	1.3	2.4	0.5	0.9	1.7
RET-RBC-X	ch	15.8	17.5	19.5						
RET-RBC-Y	ch	162	172	179						
RET-RBC-Z	ch				28.0	31.5	34.7	26.4	30.4	33.7
RET-Y	ch	170	180	188						

% - percentage of a cell population, ch - channel, FI - fluorescence intensity, SI - scatter intensity

Table 1 (B) – White blood cell related parameters

Parameters	Unit	Males and females		Parameters	Unit	Males and females		es	
		ш	Me	UL			ш	Me	UL
TNC	10 ⁹ /L	3.7	5.8	9.3	HFLC \$	10º/L	0.00	0.00	0.02
WBC	10 ⁹ /L	3.7	5.8	9.2	HFLC \$	%	0.0	0.0	0.3
WBC-D	10 ⁹ /L	3.8	5.8	9.3	NE-FSC	ch	85.5	91.2	97.4
WBC-P	10 ⁹ /L	3.7	5.8	9.2	NE-WX		291	317	345
NEUT	10 ⁹ /L	1.6	3.1	5.8	NE-WY		550	597	651
NEUT	%	37.6	54.1	69.3	NE-WZ		589	775	911
LYMPH	10 ⁹ /L	1.1	1.9	3.3	LY-X	ch	74.6	77.7	80.8
LYMPH	%	20.0	33.5	48.8	LY-Y	ch	63.5	68.6	74.2
MONO	10 ⁹ /L	0.3	0.5	0.8	LY-Z	ch	58.5	61.0	63.2
MONO	%	5.3	8.2	12.4	LY-WX		455	531	614
EO	10 ⁹ /L	0.05	0.16	0.53	LY-WY		752	870	1011
EO	%	0.9	2.8	8.3	LY-WZ		465	647	800
BASO	10 ⁹ /L	0.02	0.04	0.10	MO-X	ch	115	118	121
BASO	%	0.3	0.8	1.6	MO-Y	ch	99	109	118
BASO-D	10 ⁹ /L	0.01	0.04	0.08	MO-Z	ch	64.2	68.4	72.4
BASO-D	%	0.3	0.5	0.9	MO-WX		224	264	300
IG	10 ⁹ /L	0.01	0.03	0.07	MO-WY		534	689	861
IG	%	0.2	0.6	1.0	MO-WZ		478	780	935
NEUT-RI *	FI	42.0	46.1	50.6	EO-X	ch	182	194	203
NEUT-GI *	SI	143	149	157	EO-Y	ch	33.4	35.8	38.7
RE-LYMP *	10 ⁹ /L	0.03	0.06	0.17	EO-Z	ch	97	113	127
RE-LYMP *	%WBC	0.4	1.1	2.5	EO-WX		121	203	261
RE-LYMP *	%LY	1.3	3.3	7.8	EO-WY		383	497	623
AS-LYMP * ^{\$}	10º/L	0.00	0.00	0.00	EO-WZ		97	472	764
AS-LYMP * ^{\$}	%WBC	0.0	0.0	0.0	BA-X	ch	176	189	199
AS-LYMP **	%LY	0.0	0.0	0.0	BA-Y	ch	150	164	182
RE-MONO \$	10 ⁹ /L	0.00	0.01	0.02	BA-WX		13	117	195
RE-MONO \$	%WBC	0.0	0.2	0.4	BA-WY		13	101	300
RE-MONO \$	%MO	0.0	2.0	4.4					

% - percentage of a cell population, ch - channel, FI - fluorescence intensity, SI - scatter intensity

Table 1 (C) – Platelet related parameters

Parameters	Unit	Males and females					
		LL	Me	UL			
PLT-I	10 ⁹ /L	164	254	369			
PLT-O *	10 ⁹ /L	154	235	344			
PLT-F *	10 ⁹ /L	167	256	377			
IPF *	10 ⁹ /L	3.1	7.9	18.7			
IPF *	%	1.2	3.1	8.9			
PDW	fL	10.0	12.8	17.4			
MPV	fL	9.3	10.7	12.7			
P-LCR	%	19.3	31.2	47.1			
PCT ^{\$}	L/L	0.002	0.003	0.004			
H-IPF	%	0.3	0.9	3.0			
PLT-F-X	ch	69.7	78.7	87.7			
PLT-F-Y	ch	47.8	59.4	72.2			
PLT-F-Z	ch	39.4	44.6	50.7			

% – percentage of a cell population, ch – channel, FI – fluorescence intensity, SI – scatter intensity

Table 2 Sysmex XN-Series analyser parameter explanations

XN parameter	Explanation
AS-LYMP	Lymphocytes mainly synthesizing antibodies with high fluorescence intensity
AS-LYMP%	Percentage of lymphocytes mainly synthesizing antibodies with high fluorescence intensity
AS-LYMP, %L	The ratio of the AS-LYMP count to the lymphocyte count
BA-WX	The fluorescent light distribution width of the BASO area on the WNR scattergram
BA-WY	The forward scattered light distribution width of the BASO area on the WNR scattergram
BA-X	The fluorescent light intensity of the BASO on the WNR scattergram
BA-Y	The forward scattered light intensity of the BASO on the WNR scattergram
BASO	Basophils
BASO-D	Basophils as measured in the WDF channel
Delta-He	Difference of haemoglobin equivalent between RET and RBC
Delta-HGB	Delta-HGB is calculated by the equation HGB – HGB-O
EO	Eosinophils
EO-WX	The lateral scattered light distribution width of the EO area on the WDF scattergram
EO-WY	The fluorescent light distribution width of the EO area on the WDF scattergram
EO-WZ	The forward scattered light distribution width of the EO area on the WDF scattergram
EO-X	The lateral scattered light intensity of the EO area on the WDF scattergram
EO-Y	The fluorescent light intensity of the EO area on the WDF scattergram
EO-Z	The forward scattered light intensity of the EO area on the WDF scattergram
FRC	The absolute count and percentage calculated from the count in a specific area below the RBC area in the RET scattergram
НСТ	Haematocrit
HFLC	The count of the upper LYMPH area of the WDF scattergram
HFR	High fluorescence ratio
HGB	Haemoglobin concentration
HGB-O	Haemoglobin concentration calculated from the RET channel
H-IPF	The ratio to the total platelet count of the count of platelets that appear in the area of stronger fluorescent light intensity within the IPF area of the PLT-F scattergram
HYPO-He	The ratio of the count in the low level area of the forward scattered light signal in the RBC (mature red blood cell) area of the RET scattergram, to mature red blood cells
HYPER-He	The ratio of the count in the high level area of the forward scattered light signal in the RBC (mature red blood cell) area of the RET scattergram, to mature red blood cells
IG	Immature granulocytes
IPF	Immature platelet fraction
IRF	Immature reticulocyte fraction
IRF-Y	The intensity of forward scattered light from the IRF area on the RET scattergram
LFR	Low fluorescence ratio
LYMPH	Lymphocytes
LY-WX	The lateral scattered light distribution width index of the LYMPH area on the WDF scattergram
LY-WY	The fluorescent light distribution width index of the LYMPH area on the WDF scattergram
LY-WZ	The forward-scattered light distribution width index of the LYMPH area on the WDF scattergram
LY-X	The lateral scattered light intensity of the LYMPH area on the WDF scattergram

Table 2 Sysmex XN-Series analyser parameter explanations

XN parameter	Explanation
LY-Y	The fluorescent light intensity of the LYMPH area on the WDF scattergram
LY-Z	The forward-scattered light distribution width index of the LYMPH area on the WDF scattergram
MacroR	Macro RBC ratio
МСН	Mean corpuscular haemoglobin
МСНС	Mean corpuscular haemoglobin concentration
MCHC-O	MCHC-O is calculated by the equation HGB-O / HCT
MCV	Mean corpuscular volume
MFR	Medium fluorescence ratio
MicroR	Micro RBC ratio
MONO	Monocytes
MO-WX	The lateral scattered light distribution width index of the MONO area on the WDF scattergram
MO-WY	The fluorescent light distribution width index of the MONO area on the WDF scattergram
MO-WZ	The forward-scattered light distribution width index of the MONO area on the WDF scattergram
MO-X	The lateral scattered light intensity of the MONO area on the WDF scattergram
MO-Y	The fluorescent light intensity of the MONO area on the WDF scattergram
MO-Z	The forward-scattered light intensity of the MONO area on the WDF scattergram
MPV	Mean platelet volume
NE-FSC	The forward-scattered light intensity of the NEUT area on the WDF scattergram
NEUT	Neutrophils
NEUT-GI	Neutrophil granularity intensity
NEUT-RI	Neutrophil reactivity intensity
NE-WX	The lateral scattered light distribution width index of the NEUT area on the WDF scattergram
NE-WY	The fluorescent light distribution width index of the NEUT area on the WDF scattergram
NE-WZ	The forward-scattered light distribution width index of the NEUT area on the WDF scattergram
NRBC	Nucleated red blood cells
РСТ	Plateletcrit
PDW	Platelet distribution width
P-LCR	Platelet large cell ratio
PLT	Platelet count
PLT-F	Platelet count as measured in the PLT-F channel
PLT-F-X	The fluorescent light intensity of the PLT area on the PLT-F scattergram
PLT-F-Y	The forward scattered light intensity of the PLT area on the PLT-F scattergram
PLT-F-Z	The lateral scattered light intensity of the PLT area on the PLT-F scattergram
PLT-I	Platelet count as measured in the RBC/PLT channel
PLT-O	PLT count calculated from the RET channel
RBC	Red blood cell count
RBC-He	Mature RBC haemoglobin equivalent
RBC-O	RBC count calculated from the RET channel
RDW-CV	Red cell distribution width – coefficient of variation
RDW-SD	Red cell distribution width – standard deviation
RE-LYMP	Lymphocytes reacting to infection with high fluorescence intensity
RE-LY/MP %	The ratio of the PE LVAAD count to the lumphocute count
RE-LIMP, 70L	Absolute count of reactive monocytes
RE-MONO#	Possible could of reactive monocytes
	Peactive monocytes as a percentage of monocytes
RET.	Reactive monocytes as a percentage of monocytes
RET-He	Reticulocyte baemoglobin equivalent
RET-RBC-X	The fluorescent light intensity of RRC (mature red blood cells) area on the RFT scattergram
RET-RBC-Y	The forward scattered light intensity of RBC (mature red blood cells) area on the RET scattergram
RET-RBC-7	The lateral scattered light intensity of the RBC (mature red blood cells) on the RFT scattergram
RET-Y	The forward scattered light intensity of the RET area on the RET scattergram
RPI	Reticulocyte production index
TNC	The total nuclear cell count (WBC#+NRBC#)
WBC	White blood cell count
WBC-D	The WBC count calculated from the WDF channel
WBC-P	WBC count calculated from WPC channel