Jolien J. Luimstra, Rüya G. Koçer, Alexander Jerman, Jacqueline Klein Gunnewiek, Karlijn Gijzen, Leo H.J. Jacobs and Ayşe Y. Demir*

Current state of the morphological assessment of urinary erythrocytes in The Netherlands: a nationwide questionnaire

https://doi.org/10.1515/cclm-2020-0236
Received March 2, 2020; accepted March 28, 2020

Abstract

Background: The morphological assessment of urinary erythrocytes (uRBC) is a convenient screening tool for the differentiation of nephrological (dysmorphic) and urological (isomorphic) causes of hematuria. Considering the morphological heterogeneity, this analysis is often perceived as difficult. There is no clear (inter) national consensus and there is a lack of external quality assessment programs. To gain insight into the heterogeneity within and between laboratories, we scrutinized the current state of this analysis in Dutch medical laboratories.

Methods: The laboratories, affiliated with the Dutch Foundation for Quality Assessment in Medical Laboratories, were invited to participate in a web-based survey, consisting of two questionnaires. The first one provided information about the institution and laboratory organization, and the second explored the variability in the morphological analysis of uRBC on the basis of categorization of 160 uRBC images. Statistical analysis was premised on binomial significance testing and principal component analysis.

Results: Nearly one third of the Dutch medical laboratories (65/191) with 167 staff members participated in the survey. Most of these laboratories (83%) were an integral part of secondary care. The statistical analysis of the evaluations of the participants in comparison to the consensus (three experts from two different medical laboratories) suggested a great degree of heterogeneity in the agreement. Nearly half of the participants consciously disagreed with the consensus, whereas one fifth demonstrated a random relationship with it.

Conclusions: In Dutch medical laboratories, results from morphological analysis of uRBC are heterogeneous, which point out the necessity for standardization and harmonization.

Keywords: dysmorphic; erythrocyte; external quality assessment program; hematuria; morphology; red blood cell; urine.

Introduction

The diagnostic potential of morphological analysis of erythrocytes in urine has been recognized for decades [1, 2] and remains one of the most important tools in differentiating nephrological and urological causes of hematuria. Hematuria, which is prevalent in 2%–31% of the population and has an incidence of 6%–20% [3, 4], can present itself in many forms: it can be transient or persistent, macroscopic or microscopic, and can be accompanied by clinical complaints or can remain asymptomatic.

The underlying cause of hematuria can be broadly classified as glomerular (nephrological) and non-glomerular (urological), and the morphologic assessment of erythrocytes and cellular casts in urine has been advocated as an important non-invasive screening tool [5]. In the case of glomerular hematuria, urinary erythrocytes (uRBC) vary in size and shape, and have a multiform morphology (dysmorphic, *d*RBC), whereas in non-glomerular hematuria uRBC have a uniform morphology (isomorphic, *i*RBC). The most appropriate cut-off value for the proportion of *d*RBC in urine, above which glomerular origin is considered as the main (or most likely) cause of hematuria, varies between 20 and 80% [6–19]. In several Dutch guidelines,

***Corresponding author: Ayşe Y. Demir,** MD, PhD, Department of Clinical Chemistry and Hematology, Meander Medical Centre, Postbus 1502, 3800 BM Amersfoort, The Netherlands, Phone: 0031-33-8504344, E-mail: ay.demir@meandermc.nl
Jolien J. Luimstra, Rüya G. Koçer, Karlijn Gijzen and Leo H.J. Jacobs: Department of Clinical Chemistry and Hematology, Meander Medical Centre, Amersfoort, The Netherlands
Alexander Jerman: Department of Nephrology, University of Ljubljana, Ljubljana, Slovenia
Jacqueline Klein Gunnewiek: Section General Chemistry, Dutch Foundation for External Quality Assessment in Medical Laboratories (SKML), Nijmegen, The Netherlands

Published online April 25, 2020

the presence of ≥40% *d*RBC and/or the presence of cellular casts are included in the criteria for referral to a nephrologist [20–22]. Moreover, it has been estimated that by implementing these criteria, 25% of the patients have been spared from unnecessary extensive urological investigation [7].

To date, in many countries, including The Netherlands, urinalysis in routine clinical laboratories is performed by certified and qualified medical laboratory technicians using validated automated urinalysis systems (dipstick and urine sediment) and/or manual urine sediment microscopy (bright field and/or phase-contrast microscopy). Despite the fact that international guidelines and advances in automation have had a large positive impact on the standardization of urinalysis, several bottlenecks remain [23–25]. One of the challenges is the accuracy of many automated urine sediment analyzers with respect to their ability to recognize formed elements, i.e. *d*RBC and cellular casts [26]. Most of the recognition software fail to recognize and correctly categorize these particles, especially when numbers are low and forms are intermediate or similar (i.e. yeasts) rather than distinctive [23–26]. Therefore, most of the medical laboratories in The Netherlands rely on manual microscopy for the morphological analysis of *d*RBC and cellular casts.

In daily laboratory practice, the assessment of *d*RBC is perceived as difficult (and time-consuming) even by experienced technicians. As dysmorphism covers numerous heterogeneous morphological variations, the categorization of *d*RBC can greatly differ within and between laboratories. The lack of external quality assessment programs concerning urinary sediment further increases heterogeneity [27]. Moreover, there is no clear consensus with respect to reporting the results (and interpretations) to clinicians.

As such, variations in the morphological analysis of *d*RBC (and in the reporting of the results) are likely to result in discrepancies between and within medical laboratories and possibly in sub-optimal patient care [28]. In order to investigate the perceived heterogeneity in the analysis, interpretation and reporting of *d*RBC results, we explored the current state of morphological analysis of *d*RBC in Dutch medical laboratories. To do so, we surveyed all phases of the analytical process: pre-urinalysis (e.g. preservation), urinalysis (e.g. manual vs. automated) and post-urinalysis (e.g. reports to the clinician). Additionally, a set of high-resolution images of erythrocytes in urine was used to investigate and to compare the classification of erythrocytes by the participating laboratories.

Materials and methods

A web-based survey, consisting of two questionnaires, was sent to medical laboratories (n = 191) under the auspices of the Dutch External Quality Assessment (SKML). The laboratories were registered participants of the SKML – General Chemistry Scheme. For the first questionnaire (institutional data), specialists in laboratory medicine (EuSpLM or the affiliated quality managers) were asked to provide information about their institution and laboratory organization. The survey covered the following phases: pre-urinalysis (e.g. preservation), urinalysis (manual vs. automated) and post-urinalysis (e.g. reports to the clinician). For the second questionnaire (staff data), the laboratory staff, who were trained and qualified to perform morphological analysis of uRBC, were requested to categorize 160 images of uRBC into four categories, i.e. isomorphic, dysmorphic, acanthocytes and doubtful. The selection of 160 images was based on the consensus of three experts (one specialist of clinical chemistry and laboratory medicine, EuSpLM and two nephrologists) from two different medical laboratories, whose judgments were in concurrence with the literature [2]. The survey remained open between mid-March and mid-September 2018; reminders were sent twice.

The urine samples used to prepare the images were collected as a part of the routine diagnostic analysis and were anonymized. The images of uRBC were prepared from urine samples that were collected and prepared for microscopy as described previously [29]. Samples were analyzed using a phase-contrast Leitz Dialux 20 microscope (Leica Microsystems, GmbH, Wetzlar, Germany) at the Clinical and Research Laboratory on Urinary Sediment, U.O. di Nefrologia, Ospedale Policlinico, Milan, Italy. Digital images of high-power (400×) phase-contrast field were acquired using a mounted photo camera and processed using IrfanView (for Windows, version 4.38, 2014) to obtain high-quality images of 160 different uRBC.

Statistical analysis was based on comparing assessments of the 'laboratory staff' (henceforth 'participants') with the 'literature-based judgments of consensus of three expert opinions' (henceforth 'consensus') [2]. As two out of 160 images were not categorized by several participants, 158 images were used for the statistical analysis. For this purpose, we converted the assessments into a quantitative format by using the consensus as our benchmark as follows: if a participant's evaluation of a given picture was identical with the consensus, then this evaluation was scored 0. If the participant's evaluation differed from the consensus, then it was scored 1. In this way, the qualitative dataset that contained participants' categorization of the 158 pictures was converted into a matrix consisting only of 0 and 1 entries. This matrix was used to determine to what extent deviation or concurrence between any given participant and the consensus could be considered statistically significant. For this purpose, we estimated the probability for each participant based on the null-hypothesis that participants' evaluation on any given single picture concurred with the consensus solely due to pure chance. This null-hypothesis implied that for any given picture, the probability of agreeing with the consensus equals to 0.5. We then obtained the null probability (henceforth 'deviation score') for each participant by using the binomial formula as follows:

$$P(k) = \left(\frac{158}{k}\right) 0.5^{k} 0.5^{158-k}$$

Here, k represented the number of pictures for which a participant deviated from the consensus and thus P(k) denoted the probability of having k deviations from the consensus by pure random choice.

p-Values <0.05 were considered as statistically significant from the random answer selection. Note that both a strong agreement with consensus and a strong disagreement with consensus would have resulted in low p-values (as they both differ from pure random choice).

After this step, the deviation matrix was examined further to see whether there were clusters among the participants relative to each other beyond their concurrence with or deviation from the consensus. This analysis required examining the proximity of participants in the multi-dimensional space defined by the deviation matrix. For this purpose, we calculated the Euclidian distance between all pairs of participants. The outcome was a 167×167 proximity matrix showing how close participants were clustered together. Obviously, it was not possible to visualize the proximity picture conveyed by this matrix and thus it was necessary to reduce its dimensions. To accomplish this, principal component analysis on the proximity matrix was performed, and the space generated by the first two components was scrutinized.

Results

Questionnaire, part I, institutional data

Table 1 summarizes the data. Out of 191 medical laboratories, 65 (34%) responded to the questionnaire, and all were certified for ISO 15189.

Most laboratories (78%) implemented a stepwise reflex testing approach for urinalysis, with urine strip analysis as a first step, followed by urine sediment analysis in case of aberrant strip results. Requests for the morphological analysis of uRBC were limited to <30 requests per week in most of the laboratories (95%).

Native samples were used for urine strip (83%), sediment (86%) and *d*RBC (62%) analysis in most of the participating laboratories. Tubes with additives (i.e. VACUETTE® Stabilur [Greiner Bio-One] or BD Vacutainer® [Becton Dickinson]) were used for the analysis of urine strip, sediment and *d*RBC in 25% of the participating laboratories. Nearly one third of the laboratories used a fixative (BD CellFix™ 1–5%, Becton Dickinson) for the analysis of *d*RBC, with the prerequisite to fix samples within 30–60 min after urine production. Of these laboratories, 80% added a fixative to the urine samples after centrifugation, compared to 20% before centrifugation. Once the samples were fixed, cells and formed elements were preserved for up to 10 days.

All laboratories performed *d*RBC analysis by manual microscopy at 400× magnification (62%), or in combination (32%) with lower magnifications (100× or 200×). Forty-nine percent used phase-contrast and 38% used bright field microscopy; only few used polarized light to differentiate between lipids and crystals (13%).

Few laboratories took digital images of urine samples (26%) upon interesting findings. Digital images were obtained directly from automated sediment analyzers (16%) or from compound microscopes (10%), either using a camera permanently mounted on the microscope or using a conventional digital camera or smartphone at one ocular lens.

Forty-three percent of the laboratories with combined urine strip and sediment analyzers applied predefined rules, either at the level of the laboratory information system (LIS) or at the level of middleware. Nearly 36% of the laboratories reported their findings directly to the clinician, whereas 64% had more additional authorization steps (i.e. delta-check function within the LIS or VALAB – Werfen, The Netherlands). The analysis of *d*RBC was performed by specially trained technicians in nearly all laboratories (99%). Results were verified (independently) by a second technician (35%) or a clinical chemist (15%), meaning 50% of the laboratories performed no verification.

Questionnaire, part II, staff data

Out of 65 laboratories, 167 staff members (128 female, 39 male, between 23 and 65 years old, mean age 45 years) participated in the survey. Staff was employed as medical laboratory technicians for routine (50%) or specialized analysis (13%). Twenty-eight percent of participants were specialists in laboratory medicine (EuSpLM), whereas 9% did not state their position.

Ninety-nine percent of the participants followed an in-house training (of which 60% conform to ISO 15189) for routine urine strip and sediment analysis, and morphological analysis of *d*RBC. Eighteen percent of participants also followed an extramural training and 1% were self-educated using literature and attendance at symposia. Ninety-nine percent of the participants considered themselves qualified to conduct strip and sediment analysis, and 87% felt qualified in conducting morphological analysis of *d*RBC. The necessity for continuous training in urine analysis was acknowledged by 85% of the participants, whereas the 3% filled responded with ‘maybe’ and 12% with ‘no’.

Figure 1 shows the comparison of the participants’ evaluations with the consensus, as well as three representative images for each *d*RBC category included in the questionnaire. Erythrocytes ranked isomorphic by consensus

Table 1: Summary of the data from questionnaire part I (institutional data).

(A) Questions related to pre-analysis				
Type of laboratory organization				
Primary care (general practitioners)		3%		
Secondary care (regional hospital)		83% (63% of these laboratories also supported primary care)		
Tertiary care (academic hospital)		14%		
Number of requests per week for		**Urine strip**	**Urine sediment**	**Dysmorphic erythrocyte**
Strip or sediment	Dysmorphic			
<200	<10	41%	71%	59%
200–400	11–20	22%	22%	21%
400–600	21–30	22%	3%	15%
600–800	31–40	10%	2%	5%
>800	>50	5%	2%	0%
Preservation of urine samples		**Native**	**Additive**	**Fixative**
Not applicable		14%	75%	71%
Strip and sediment		24%	5%	0%
Sediment and dysmorphic		3%	11%	3%
Strip, sediment and dysmorphic		59%	9%	0%
Dysmorphic		0%	0%	26%
Transport of urine samples within		**Urine strip-sediment**		**Dysmorphic erythrocyte**
1 h		8%		48%
2 h		23%		21%
4 h		50%		31%
6 h		3%		0%
8 h		8% only if refrigerated		0%
24 h		8% only if refrigerated		0%
(B) Questions related to analysis				
Urinalysis workflow				
No strip analysis		4%		
Manual strip analysis		7%		
Automatic urine strip analyzer		89%		
No sediment analysis		2%		
Manual urine sediment analysis		58%		
Automatic urine sediment analyzer		40%		
Vendor for urinalysis			**Type of instrument**	
Roche Netherlands B.V.		32%	Cobas Urisys 411, 601, 701, 2400, 6500	
Sysmex Netherlands		32%	UC-1000, UC-3500, UF-500i/1000i, UF-5000/4000, UX-2000, one lab UD-10	
Beckman-Coulter Netherlands		18%	iCHEMVelocity, iQ200 Elite	
Menarini Netherlands		12%	AutionMax and SediMax	
Siemens Netherlands		6%	Clinitek Advantus, Atellica	
Type of manual microscopy				
Phase-contrast		49%		
Bright field		38%		
Polarized light (if needed for lipids, crystals)		13%		
Usage of magnification				
Low (100×)		5%		
High (400×)		62%		
Low (100×), high (400×)		25%		
Low (200×), high (400×)		5%		
Low (100×), low (200×), high (400×)		2%		
(C) Questions related to post-analysis				
Registration of the images in the electronic patient file and authorization of the results		See the text		

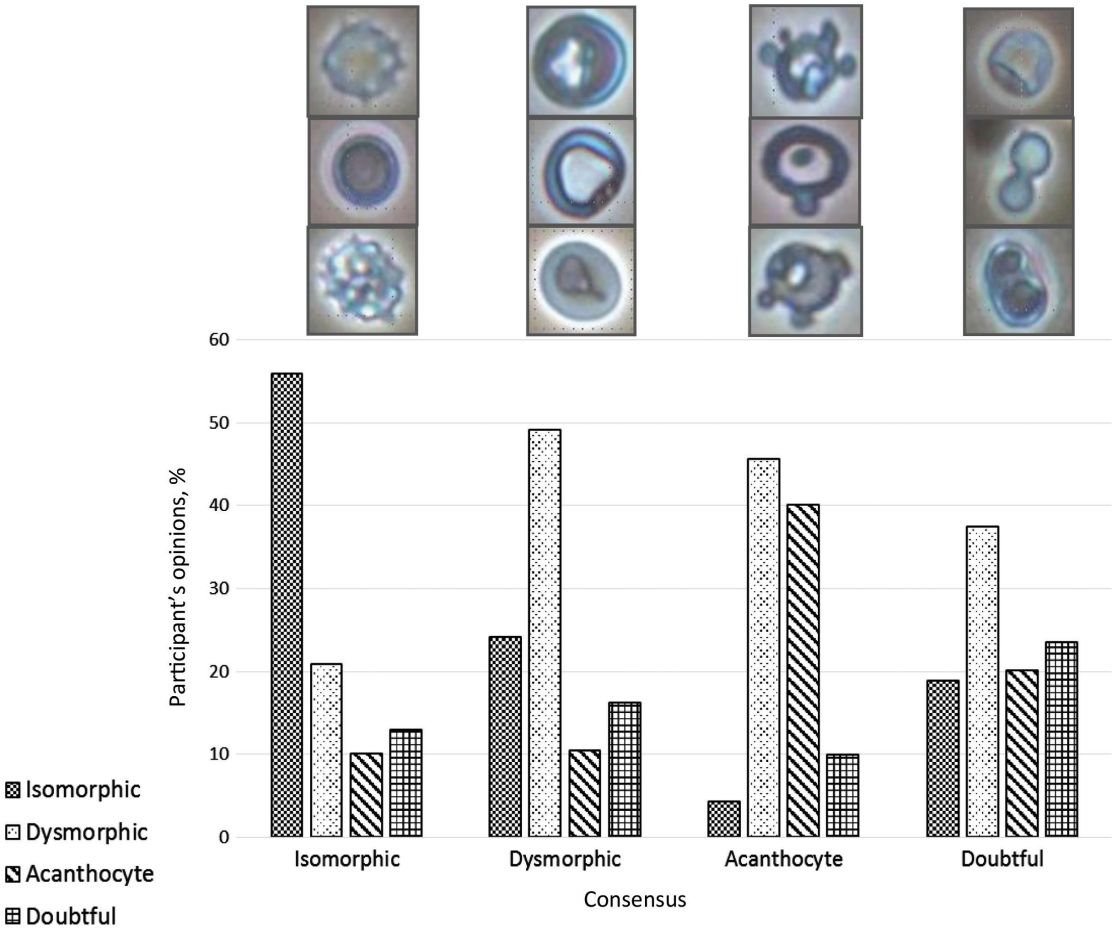

Figure 1: Conditional distributions generated by keeping the consensus constant and capturing variation in opinions.

were concurrently ranked isomorphic by 56% of the participants, compared to 21% dysmorphic, 10% acanthocyte and 13% doubtful. Erythrocytes considered dysmorphic according to consensus were ranked isomorphic by 24% of the participants, dysmorphic by 49%, acanthocyte by 11% and doubtful by 16%. These numbers shifted to 4% isomorphic, 46% dysmorphic, 40% acanthocyte and 10% doubtful in the case of acanthocytes, whereas for doubtful pictures the distribution of assessments was 19% isomorphic, 37% dysmorphic, 20% acanthocyte and 24% doubtful.

The binomial analysis indicated that having less than 70 or more than 90 disagreements with the consensus implied that the participant's conformity with the consensus could not be explained by pure chance and must have been based on deliberate choice. Following this reasoning, less than 70 disagreements implied conscious (i.e. statistically significant) agreement and more than 90 implied conscious (i.e. statistically significant) disagreement with the consensus. Scores between 70 and 90, on the other hand, implied that a participant's conformity with the consensus might be due to pure chance. In this survey, 58 participants (35%) belonged to the group of conscious agreement with the consensus, 74 participants (44%) belonged to the group of conscious disagreement with the consensus, and 35 participants (21%) belonged to the group of random evaluations with the consensus.

In a further elaboration on these results, we studied whether clear clusters among the participants relative to each other (beyond their concurrence with or deviation from the consensus) were present. It turned out that the first two principal components approximately accounted for 80% of the entire variance (PC1 70% and PC2 9%). Of these, the first component largely coincided with the deviation scores of participants (correlation of –0.95). Therefore, it might have been taken as the opposite of the distance between the consensus and any given participant: the higher the score of a participant on the first component, the closer he/she should have been to the consensus. We interpreted the second component as a complementary measure that showed the concurrence between participants in terms of how similar they were in evaluating the same pictures.

The closer the participants were in their exact deviations or agreements on more pictures, the closer they were located in the scale defined by the second component.

In Figure 2, the scatter of participants in the two-dimensional space defined by these two components is shown. Here, the star is the consensus point. Circles (blue) represent those who consciously agree with the consensus, triangles (red) depict those who consciously disagree with the consensus, and crosses (black) show the participants whose choices could not be distinguished from random selection with the consensus. As one would have expected, the conscious agreement and conscious disagreement groups were nearly separated and the random-choice group appeared as the cushion in between them. However, it is important to note that there were clusters in the space defined by the principal components characterized by close proximity points. This suggested that there were sub-groups of participants with similar reasoning behind their evaluations.

This point was further elaborated in Figure 3. Here, the scatter in Figure 2 was reproduced, but these time points that represented those participants who were affiliated with the same organization were shown with special icons (pluses, crosses, triangles and pentagons) if they were at least eight, while the remaining participants were given once again as dots. The expectation that we examined was that people who worked in the same environment would have been more inclined to develop common perceptions and evaluation practices and thus would have appeared close to each other in the two-dimensional space defined by principal components. In fact, as one can see in Figure 3 in the case of the organization depicted by pluses, all participants appeared in close proximity, and the same was true for the organization represented by triangles. In the case of the other two organizations (depicted by crosses and pentagons), at least half of the participants (i.e. four) appeared quite close to each other. This entire picture suggested that in the absence of a single standard, there

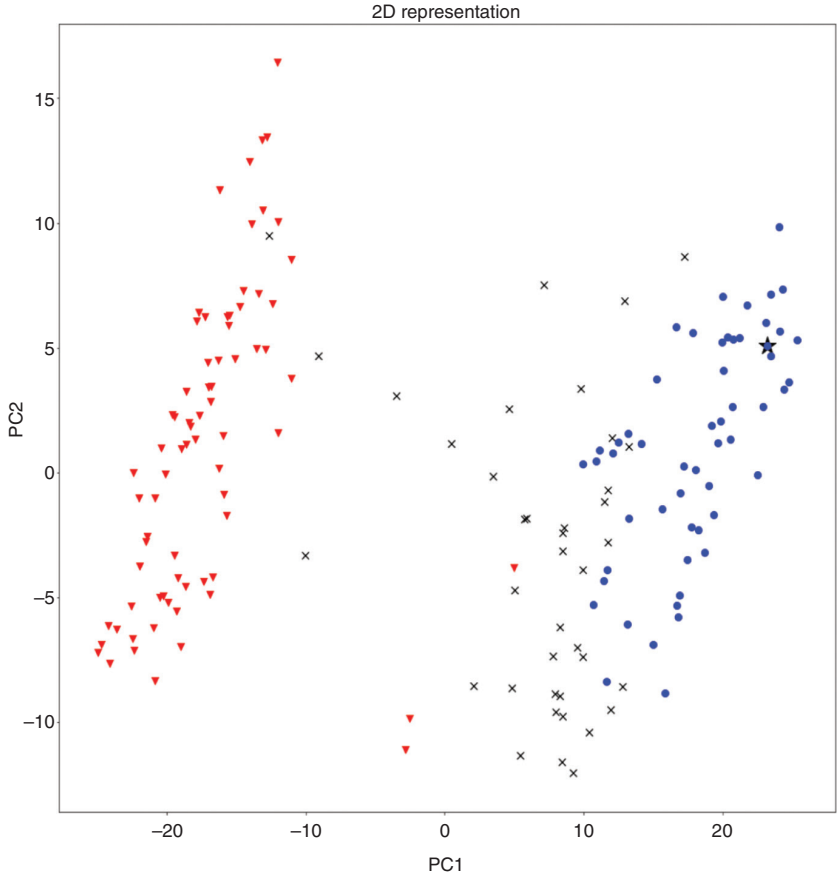

Figure 2: Conscious agreement, disagreement and random deviations from the consensus in the two-dimensional space defined by the first two principal components derived from the proximity matrix.
The star is the consensus. Circles represent those who consciously agree with the consensus, triangles depict those who consciously disagree with the consensus, and crosses show the participants whose choices could not be distinguished from random selection vis-à-vis consensus.

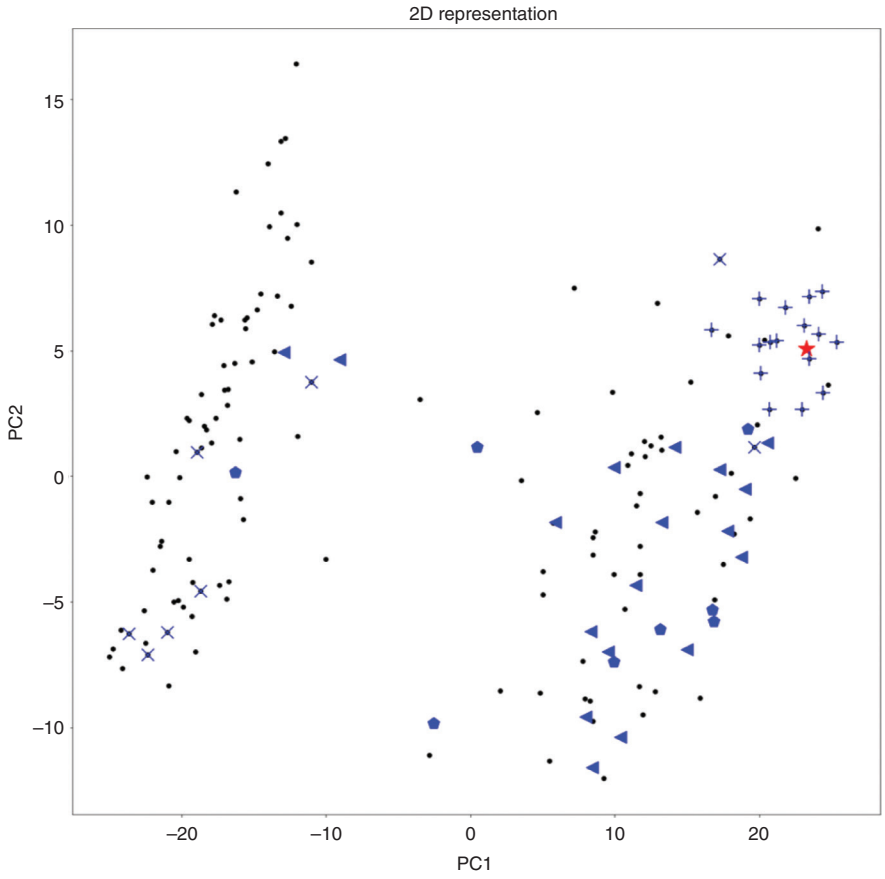

Figure 3: Clustering of opinions in the two-dimensional space defined by the first two principal components derived from the proximity matrix.
Participants from the same organizations are depicted by the special icons (crosses, pluses, triangles and pentagons) if they exceed 7 in the survey. The remaining participants are all shown as dots. The participants depicted by 'crosses' belong to the same laboratory organization at which one of the experts (EuSpLM) works. These participants assured that they independently categorized images in the survey at separate time points.

seemed to have developed distinct knowledge communities that reached similar judgments within themselves.

Discussion

Of the multiple studies that have evaluated the usefulness of morphological analysis of uRBC, some show added benefit [1–2, 5, 13, 30, 31], while others have found it to be of little added value [12, 32–34]. There are only a limited number of studies on the discovery of alternative urinary biomarkers and their function as triage tests. These studies have provided limited evidence of diagnostic performance to justify the routine use of these markers in the evaluation of hematuria, and prospective studies are needed [35–40]. Nevertheless, the clinical relevance of *d*RBC in recognizing glomerular hematuria is widely accepted and implemented [20–22, 41]. Although the morphological analysis of uRBC has a prominent role as a starting point for workup to identify the underlying pathology of bleeding, little is known about variations within and between laboratories.

In this study, we have found large variation within and between Dutch medical laboratories. The comparison of the evaluations of the participants with the consensus showed remarkable observations. The category classified as isomorphic according to the consensus was judged differently by 44% of the participants, of which 31% ranked them dysmorphic or acanthocyte. Subsequent inspection of these results suggested that these differences were mainly seen with images of crenated or ghost erythrocytes, or pseudo-G1 cells (bite cells). These findings are to a large extent in concordance with two previous reports: a Brazilian external quality assessment (EQA) program on dysmorphic erythrocytes [27] and an

Italian EQA program on urinary sediment [42, 43]. In the Brazilian EQA program (five rounds in 13 months), 83% of the participants had correctly identified isomorphic erythrocytes in round 1 and 93% in round 5 [27]. In the Italian EQA program, a similar percentage of correct identifications has been described, i.e. 91.5%, 63.9% and 77.7% in the first, second and third structure presentation, respectively [36], or 80.7% ± 24.7% (presented 3 times) [43]. Both programs confirm that EQA programs improve participants' knowledge.

The Brazilian EQA program reported a rate of 58.5% in rounds 1, 2 and 3 to 76.2% in rounds 4 and 5 for dysmorphic erythrocytes, whereas for acanthocytes it was 66.4% to 84.2%, respectively [27]. The Italian EQA described the rates of correctly identified dysmorphic erythrocytes as 86.9%, 90.2% and 97.5% in rounds 1, 2 and 3, respectively [36], or as 77.0 ± 8.1% of the participants (presented twice) [43], whereas the correct identification rate for acanthocytes has been found lower in the first round by 72.8% [42] and 78.2% (presented once) [43]. A similar discrepancy was observed in this study: in ranking of the consensus-based dysmorphic category, 24% of the participants categorized it as isomorphic. Additional manual inspection of the results revealed that these discrepancies could be traced back to images of erythrocytes with membrane or cytoplasm irregularities. It is important to note that the best agreement was found in classifying the clinically important acanthocytes: 86% of the participants agreed that the shape of these erythrocytes was aberrant (acanthocyte and dysmorphic).

Statistical analysis showed a great degree of heterogeneity in comparison to the consensus. Interestingly, the group of participants consciously disagreeing with the consensus constituted the relative majority (44%), whereas only 21% of the participants demonstrated a random relationship with the consensus. This outcome implied that the consensus did not occupy a significant position in the evaluation process of participants. Obviously, this did not necessarily mean that participants within the conscious or random deviation groups agreed among themselves; two participants might deviate from the consensus but might still disagree with each other. A possible (somewhat pessimistic) interpretation could be that there was no standard evaluation of uRBC morphology. Fortunately, the visualization based on our principal component analysis showed that within each broad category (conscious agreement, disagreement and random choice), there were small groups of participants clustered among themselves. On the basis of this finding, one might argue that in the absence of a single standard there seemed to have appeared distinct knowledge communities that reach similar judgments within themselves.

In this study, we took advantage of accessibility to all medical laboratories that were part of SKML – General Chemistry Scheme in The Netherlands. By means of a web-based survey, the current state of this analysis in Dutch medical laboratories was explored, so that we could get an insight into the laboratory work flow and variation within and between laboratories. This approach comprehended several limitations. First, the validity and reliability of the two questionnaires were not evaluated beforehand in our laboratory or elsewhere. The questionnaires consisted of close-ended questions with a (bounded) continuous response scale. This type of questioning might have some disadvantages, for example respondents might have selected answers similar to true response, even though it was different. On the other hand, as all participants were asked exactly the same questions in an identical format and responses were recorded in a uniform manner, it was consistent and comparable. Secondly, the participation rate (34%) was less than expected and mainly laboratories that supported primary and secondary care, and not tertiary care, responded. Nevertheless, this rate represented, to the best of our knowledge, the largest comparison of microscopic analysis in hematuria. This might be explained by the necessity for standardization and harmonization in these type of medical laboratories and the availability of participants to fill the questionnaire.

This study clearly demonstrates variability among analysis of dysmorphic erythrocytes in Dutch medical laboratories not only in the logistic, pre-analytical and analytical handling, but also in the classification of erythrocytes. That fact that over 40% of participants showed results that consciously disagreed with consensus suggests that there is a great opportunity for improvement and that there is a need for clear and uniform guideline, uniform training and for an external quality control program to maintain uniform standardized results among laboratories.

Conclusions

The morphological assessment of uRBC is considered to be the most distinctive screening tool for the differentiation between the nephrological and urological causes of hematuria. However, the recognition and categorization of morphological deviations is not standardized and is difficult. To validate the presumption that large differences exist within

and between laboratories, a survey for Dutch medical laboratories is designed. The results show a large variability not only in laboratory process, but also in recognition and categorization concerning the morphology of uRBC. This study underscores the need for standardization and provides a valuable overview of which items require standardization.

Acknowledgments: The authors thank Prof. Dr. Giovanni B. Fogazzi and Dr. Giuseppe Garigali (Research Laboratory on Urine, Nephrology Unit, Fondazione IRCCS, Ospedale Maggiore Policlinico Mangiagalli e Regina Elena, Milano, Italy) for their contribution to the questionnaire, including the preparation of the images.
Author contributions: J.J. Luimstra, manuscript preparation; A. Jerman, concept and study design, data collection; R.G. Koçer, data collection and analysis; J. Klein Gunnewiek, concept and study design; K. Gijzen, manuscript preparation; L.H.J. Jacobs, manuscript preparation; A.Y. Demir, concept and study design, conduction of experiments, data analysis, manuscript preparation. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: Authors state no conflict of interest.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: None.

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