Image analysis of neutrophil nuclear morphology: Learning about phenotypic range and its reliable analysis from patients with Pelger-Huët-Anomaly and treated with Colchicine

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Running headline: granulocyte nuclear morphology

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Figures 5
Tables 7

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Abstract

The nuclear morphology of neutrophils depends on different endogenous and exogenous factors, which can lead to hypo- or hypersegmentation of the normally 2-4 segmented nucleus. Hypossegmentation can be due to mutations in the LBR-gene (Pelger-Huët-Anomaly) or can be induced, for example, by colchicine treatment. The range of this phenotypic variation is known as “norm of reaction”, which can be of major relevance for clinical diagnosis and therapeutic intervention. In this project, we studied the “norm of reaction” in 26 subjects with 0-3 wild type LBR alleles. In addition, the phenotypic variation was analyzed in 3 patients with Familial Mediterranean Fever (FMF), before and after colchicine treatment. We measured the phenotype nuclear segmentation of neutrophils based on two conventional qualitative methods, the “rule of threads” and the “rule of thirds”. In addition, we tested a morphometric quantitative approach, the “circularity index”. The circularity index” was superior in cases with hypossegmentation; the “rule of thirds” with respect to hypersegmentation. Approximately 65% of the observed phenotypic variance was explainable by the number of LBR wild type alleles. The gene-dosage effect followed a non-additive, hysteresis-like characteristic with lower and upper plateaus. Colchicine treatment had a clear, although minor phenotypic effect compared to the number of LBR wild type genes or the mutation type. Thus, the nuclear morphology of granulocytes and its “norm of reaction” can be regarded as an excellent model both for detailing the interplay between endogenous and exogenous factors and for clinical diagnostic purposes.

Key terms

Nuclear lobulation, neutrophils, LBR-gene, norm of reaction, hypersegmentation, hypossegmentation, colchicine
Introduction

The nucleus of almost all human cells is ovoid. An exception is the highly deformable, lobulated nucleus of neutrophils, allowing for rapid diapedesis through vessel walls. It is an evolutionary ancient trait, which is due to the paucity of lamins A/C and B1 and the elevated levels of the lamin B receptor (LBR). In addition, microtubule integrity is another requirement for its unusual nuclear morphology (1). Compared with other morphological traits, the pathway that connects the genotype with the phenotype is less complex, but still modified by endogenous and exogenous factors. Thus, abnormal lobulation is indicative of various clinical conditions. Hypersegmentation is associated with vitamin B12, folic acid, and iron deficiency and cases of myelodysplasia (2,3). Hypossegmentation is a transient phenomenon in inflammatory processes and myelodysplastic syndromes. Moreover, it can be due to side effects of drugs, such as ibuprofen or valproate (4). Already in 1953, Harm had shown that intra venous application of colchicine to rabbits leads to a temporal hypossegmentation of neutrophil nuclei (5). A hereditary cause of hypossegmentation is the Pelger-Huët-Anomaly (PHA), an autosomal dominant trait, which is caused by mutations in the LBR-gene (6).

Recently, we demonstrated a highly significant correlation between the number of LBR-genes (or the type of LBR mutation) and the degree of nuclear segmentation (7). Consequently, neutrophil nuclear lobulation is an ideal model for the analysis of the “norm of reaction”, which is based on the theoretical concept that a specific phenotype may have a range of manifestations, not only among subjects of a single genotype, but also within every single subject (8). In some cases, like human blood groups, the range of phenotypes is strictly related to the genotype, and the environment has little effect. For other phenotypes, like height in humans, the norms of reaction are much wider (9). The “norm of reaction” constitutes a probability distribution (not necessarily normally distributed) in the case of quantitative traits. Analyses of such distributions provide valuable insights of how the function of a gene is actually modulated by endogenous and exogenous factors among subjects of a pre-specified population (10), thus being of major relevance for both clinical diagnosis and therapeutic intervention.

In this study, we investigated the “norm of reaction” with respect to the degree of nuclear segmentation in neutrophils, both in individuals with 0 to 3 wild type LBR-genes and different kinds of mutations, as well as in patients with Familial Mediterranean Fever (FMF) before and after oral colchicine therapy, and also after in vitro colchicine treatment. Clearly, these analyses depend critically on the precision with which the
The conventional way to assess neutrophil nuclear lobulation is by counting the number of nuclear segments. There are two different definitions of “segment”, the “Fadenregel” (rule of threads) and the "Drittelregel" (rule of thirds). In the first case, a segment is defined by lobes which are connected only via a thin chromatin thread; in the latter case, by an indentation whose width is less than 1/3 of its length (11,12). This optical classification is based on subjective criteria and depends on the experience of the observer. Moreover, it does not consider any morphological characteristics of the counted segments (Fig. 1).

We, therefore, applied a more objective method to characterize neutrophil nuclear lobulation by measuring the perimeter of the nucleus (as an indicator of lobulation) in relation to the nuclear area; the "circularity index". Such a computer-assisted assessment of nuclear shapes was previously used on lymphocytes to detect lymphoid neoplasms and on prostatic cells to detect malignant cells (13-15). Thus, we compared the two conventional optical classifications of neutrophils with a computer-assisted, morphometric analysis of the nuclear shape.

Material and Methods
The local ethical committee approved the study. Written informed consent was obtained from all participants.

Subjects
We examined the encoded blood smears from 26 individuals with 0 to 3 wild type LBR genes. For clinical and molecular details, see (6,7). In brief: One proband is homozygous for a splice site mutation (IVS12-5-10del); another for a regulatory mutation. In both cases, some LBR protein can still be detected in western blots, with higher amounts in the case of the regulatory mutation. Among the 9 heterozygous individuals, 5 had the same splice site mutation (IVS12-5-10del) and 4 had a null mutation (2 with the stop mutation c.1129C>T; p.R377X, one had the stop mutation c.265C>T; p.R89X, and one had a deletion resulting in a premature stop codon c.32-35del4; p.V11E fs X14). In addition, 12 controls were homozygous for the wild type LBR allele. Hyperlobulation due to germline duplication of the LBR gene is extremely rare. Nonetheless, we could include 3 individuals, each with 3 LBR gene copies due to unbalanced translocations.
46,XY,dup(1)(q32q44); 46,XY,der(1)(1qter→1q42.3::1p36.3→1qter) and 46,XY,der(3)(3qter→3p26.1::1q25→1qter), respectively.

In addition, we analyzed the blood smears of 3 patients with FMF, before and after colchicine treatment. The intervals between the first and the second blood smear and the genetic status of the patients are listed in Table 1. All patients showed the typical FMF-symptoms with periodic attacks of fever and diffuse abdominal pain before colchicine treatment. Two patients were homozygous for the M694V mutation in the MEFV gene; one was compound heterozygous for the M694V mutation and the E148Q variant. Under treatment with colchicine, all three were free of FMF attacks. This is paralleled with a reduction of the C-reactive protein (CRP; Table 2). The in vitro test of colchicine was performed on blood from a normal female individual.

Methods

Nuclear segmentation was scored on photographs of 50 randomly selected neutrophil nuclei of each individual and the lobulation index was calculated by three methods: The “rule of threads”, the “rule of thirds”, and the “circularity index” (Fig. 1). The dimensionless “circularity index” is defined as $P^2/A \times 4\pi$, with nucleus perimeter $P$ and nucleus area $A$. The index value of 1 indicates a perfect circle and values above 1 reflect the number of lobes. All analyses for the study were performed by one individual (N.S.) on coded slides. Although this might be considered a weakness of the current study, evaluations performed prior to this study had demonstrated complete concordance of results obtained by the first author (N.S.), the senior author (K.H.) and an experienced hematology/oncology technical assistant (data not shown).

The preparations were analyzed on a light microscope (Leica DMRB), connected to a digital camera (AxioCam Mrc; Carl Zeiss MicroImaging GmbH) and the imaging software (AxioVision Rel. 4.5; Carl Zeiss Imaging Solutions GmbH, 2005). The nuclei were manually encircled. The perimeter and the area were calculated automatically. Part of the “rule of thirds” analysis has already been reported (7).

The in vitro colchicine test was performed on blood from an adult, healthy female. Heparinized whole blood was treated with 0.02 µg/ml colchicine for 10 and 12 hours while in an incubator at 37% C. The control blood smear was prepared after 10 hours of incubation without colchicine. 50 neutrophil nuclei were analyzed per condition and time-point.
The statistical analyses were based on 50 nuclei per condition with phenotypic variations being represented through mean values ± standard deviation. The statistical significance of differences between phenotypic distributions were analysed by the nonparametric Wilcoxon-Mann-Whitney U test (http://elegans.som.vcu.edu/~leon/stats/utest.cgi). Additionally, we fitted “Generalized Linear Models” (GLM) to the observed phenotypic data in order to model "within-subject" and "between-subject" phenotypic variation (analysis of variance). These analyses were carried out by means of the statistics package SAS 9.3 on a 64 Bit Windows system (standard procedures in combination with PROCs GLM, DISCRIM, and REG, along with nonlinear least squares approximation).

Results

Analysis of our data readily revealed a broad phenotypic range of LBR expression for all three quantification methods. The empirical within-subject phenotype distributions, as derived from 50 randomly selected cells per subject, were found to be approximately normal (individual “norm of reaction”). With the number of wild type LBR copies the “width” of the distributions increased, as each wild type gene contributes a certain amount to the overall variation (Fig. 2). The LBR gene affects nuclear lobulation in a nonlinear (non-additive) way. Taking the phenotype difference between 1 and 2 functional LBR copies as reference, it becomes readily clear that there is kind of saturation in the case of more than 2 functional LBR copies, while in the case of 0 functional LBR copies a biological “zero” is impossible for, at least, one major reason: Western blots of the subjects with no wild type LBR copies revealed detectable levels of LBR protein (7). This finding was equally true for all three methods (Table 3).

So-called “forest plots” were used to disentangle within-subject and between-subject differences in phenotype variation as a function of wild type LBR copies. In these plots, the “norm of reaction” is represented for all 26 subjects as a graph that reflects its phenotypic mean ± standard deviation. Due to the small variation inherent in the “rule of threads”, “forest plots” could only be constructed for the “rule of thirds” (Fig. 3a), and the “circularity index” (Fig. 3b). The two approaches yielded roughly the same picture: (1) individual mean values fluctuated to some extent around group means; (2) the LBR gene affected cell segmentation in a nonlinear (non-additive) way; (3) there were a few subjects deviating to a larger extent from their group means; (4) groups were consistently separated from each, except a few “outliers”.

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The amount of variance that is explainable through the \textit{LBR} genotype depends on the quantification method (Table 4). The “optimal” phenotype measure, the “circularity index”, indicated that approximately 64% of the observed phenotypic variance was explainable by the \textit{LBR} genotype when the “norm of reaction” was included in the model. This means that nuclear segmentation was not exclusively determined by the \textit{LBR} gene. Other factors such as measurement errors, environmental “noise”, as well as hidden endogenous and exogenous factors, accounted for as much as 36% of observed phenotypic variance.

In the next step, we modeled the non-linearity of the \textit{LBR} phenotype-genotype relationship based on the “rule of thirds” method and included the amount of \textit{LBR} protein, as derived from 7 lymphoblastoid cell lines (Fig. 4). It becomes obvious, that (1) the amount of observable \textit{LBR} protein, though strongly correlated with the number of wild type \textit{LBR} copies, follows non-additive, hysteresis-like characteristics with lower and upper plateaus; and (2) the phenotype quantification method assesses these nonlinear characteristics very well.

In parallel, we compared the phenotype differences between these groups by the nonparametric Wilcoxon-Mann-Whitney U test and reached statistical significant differences under all three assessment methods (Table 5). There was one exception, however: Based on the “rule of thirds”, the difference between the homozygous individuals with the splice site mutation, compared to the regulatory mutation did not reach statistical significance. The discrimination between the groups with a high lobulation index was better after application of the “rule of thirds” than the “circularity index”; whereas at low lobulation, the “circulatory index” was superior compared to the “rule of thirds”.

Apart from \textit{LBR} gene dosage effects on the lobulation index, we also tested whether these three methodological approaches can resolve subtle phenotypic changes induced by an environmental factor: Colchicine application \textit{in vitro} and \textit{in vivo}.

After colchicine application \textit{in vitro}, a significant decrease in the lobulation index was found after 10 hours (which is still evident after 12 hours), both after application of the “rule of thirds” or the “circularity index”. By contrast, this difference is not significant after applying the “rule of threads” (Table 6).

All three FMF patients showed a lower lobulation index after colchicine treatment based on the “rule of thirds” and the “circularity index”. The reduction reached statistical significance in patient “51”, but not in patients “52” and “53”. The “rule of threads” did not
reveal such differences (Table 7). It should be noted, however, that the influence of colchicine on nuclear segmentation is small compared to that of the \textit{LBR} genotype. The \textit{LBR} data indicated that all 3 FMF patients possessed 2 wild type \textit{LBR} alleles.

The higher discrimination power of the “rule of thirds” and the “circularity index” compared to the “rule of threads” became evident through their much better resolution when detailing the effects of the \textit{LBR} genotype and colchicine treatment. This is by construction, since the “circularity index” as a metric quantity ranges from 1.14 to 7.78, thus providing a much better resolution regarding the assessment of subtle differences than the other methods. This is particularly true in the lower phenotypic range. A comparison between the number of lobes based on the “rule of thirds” and the corresponding values calculated by the “circularity index” shows a nearly linear relationship up to 5 lobes. Nuclei with 6 segments, however, have a lower “circularity index” (Fig. 5), illustrating the superior resolution power of the “rule of thirds” in nuclei with hypersegmentation.

\textbf{Discussion}

The lobulated nucleus of neutrophils is exceptional considering its peculiar segmented, not ovoid, nuclear morphology. It is also exceptional both with respect to its predictive value for various clinical conditions and with respect to its analytical value in the assessment of genotype-phenotype correlations. Generally, the analysis is made by biomedical technologists under the microscope by counting the number of nuclear segments according to the “rule of threads” and the "rule of thirds". This rather subjective classification is also limited by the efficiency of the investigators and their medical knowledge, but also with respect to its discrimination power. Interestingly, almost all reports of pseudo PHA (hyposegmentation) were described before the mid 1980´s. This has been explained by the replacement of visual examination of blood smears by automated leucocyte counting which do not reveal these morphological changes adequately (16). However, the finding of hyposegmentation is of considerable clinical relevance, as has been outlined above and also with respect to the use of freely available drugs, such as ibuprofen (16). There is a need for a simple and reliable test for estimation of neutrophils’ nuclear changes. As illustrated here, in case of hyposegmentation the “circularity index” is the method of choice. This index is “1” in round nuclei and based on the assumption that the area is directly correlated to the DNA content which is the same in all neutrophil nuclei. However, the “rule of thirds” is superior.
to the “circularity index” in case of hyperlobulation. One explanation is that hypersegmented nuclei can exhibit partially overlapping segments which can be recognized visually but not by measuring the perimeter. Moreover, it cannot be excluded that heterochromatinization is more extensive in hyperlobulated nuclei, leading to a smaller nuclear area and consequently to a lower “circularity index” (1,17). Thus, the new methods for fully automatic registration of neutrophil’s nuclear shape (18-21) should combine both lobe counting according to the “rule of thirds” and calculation of the “circularity index” based on the automatically reconstructed boundaries of the lobes (nuclear perimeter) and their area. In the first case, it is the link to the clinically well established nuclear classification of neutrophils. In the second case, it adds the higher discrimination power of variance analysis which is of special interest for research purposes. Clearly, automatic neutrophil recognition on blood smears is still a challenge. Alternatively, the combination of leukocyte analysis by flow cytometry (22,23), in combination with automatic lobulation diagnosis by the “rule of thirds” and the “circularity index” could be a promising approach.

This study provided ample evidence that detailed analysis of nuclear phenotype variations, both within-subject and between-subject, can provide valuable information not only with respect to the fundamental understanding of the distinct contributions from various sources, but also with respect to clinical diagnosis and therapeutic intervention. In general, the phenotypic variation is primarily determined by the genotype-phenotype “distance” — the larger the distance the wider the range. Here, the term “distance” means the multitude of processes between the molecular and the phenomenal level. Only in rare cases, such as the qualitative differences in blood groups, is a one-to-one correspondence between a specific phenotype and a certain genotype. In most quantitative traits, such as height and body weight, the transition from genes to phenotype is of enormous complexity. Consequently, single genes explain only a minor part of the observed phenotypic variation since the function of the respective gene is typically modulated by numerous endogenous and exogenous factors (24). In this context, the nuclear phenotype of neutrophils is exceptional. The “distance” between the LBR gene, coding for a protein of the inner nuclear membrane in neutrophils, and the relevant phenotype, nuclear lobulation, is modest. Moreover, based on the “circularity index”, this phenotype is treated as a quantitative trait, allowing a rather subtle calculation of its “norm of reaction”. Here, we found that the LBR genotype explained roughly 64% of the observed phenotypic variance (nuclear segmentation), when the
gene’s “norm of reaction” was included in the model; while measurement errors, environmental noise, or hidden endogenous and exogenous factors of unknown nature, seemed to account for the unexplained 36%.

Apart from LBR also the microtubular network controls the nuclear shape of neutrophil nuclei. This was already demonstrated in 1953 by Harm after injection of colchicine in rabbits and also after application of nocodazole during in vitro granulopoiesis (25,26). Both colchicine and nocodazole disrupt the microtubular network. Here, we demonstrate an immediate effect after colchicine application in vitro as well as after colchicine treatment in vivo of patients with FMF. This effect was significant in the patient with the longest exposure of more than 200 days. Colchicine is the drug of choice for preventing attacks of fever in FMF patients (27). Interestingly, the usual doses of colchicine do not result in a visible derangement of microtubules in neutrophils (28). The effect on the lobulation index, as shown here, is therefore a more sensitive indicator of this exogenous agent. Altogether, the colchicine effect caused by therapeutic intervention is small compared to the influence of the LBR genotype.

Single case analyses, addressing the inter-individual differences of the “norm of reaction”, showed that subjects with a too large deviation from “their” standard can readily be identified, so that additional clinical assessments can be initiated where necessary. For example, 2 homozygous patients of our sample with no wild type LBR allele displayed significant differences in terms of nuclear segmentation, while a subject with 1 wild type LBR copy showed remarkable similarity with one of these cases (Fig. 3). Single case analyses also demonstrated that the amount of observable LBR protein, though strongly correlated with the number of wild type LBR alleles, followed non-additive, hysteresis-like characteristics with lower and upper plateaus. The latter has been explained by a partial dosage compensation mechanism (7).

Our LBR sample can be regarded as an excellent example for detailing the interplay between genetic factors on the one hand, and measurement errors, developmental noise, endogenous and exogenous factors on the other. Specifically, we learned from our data that (1) analysis of the “norm of reaction” can be a powerful tool for diagnostic purposes and as indicator of clinical change; (2) analysis of the “norm of reaction” can resolve subtle gradations of phenotypic heterogeneity; (3) reference values are required that enable direct classification of patients and allow one to decide upon the significance of observed deviations.
References


Acknowledgement

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### Table 1

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age [y]</th>
<th>Sex</th>
<th>Genetic status FMF</th>
<th>Colchicine treatment</th>
<th>Duration [d]</th>
<th>Dosage [mg/d]</th>
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<td>51</td>
<td>3</td>
<td>male</td>
<td>Homozygous M694V</td>
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<td>203</td>
<td>0.5</td>
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<tr>
<td>52</td>
<td>17</td>
<td>female</td>
<td>Compound heterozygous E148Q/M694V</td>
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<td>14</td>
<td>1.0</td>
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<tr>
<td>53</td>
<td>15</td>
<td>male</td>
<td>Homozygous M694V</td>
<td></td>
<td>73</td>
<td>1.0-1.5</td>
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### Table 2

<table>
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<th>Patient ID</th>
<th>Time point</th>
<th>Clinical status</th>
<th>CRP [mg/l]</th>
<th>Neutrophils [%]</th>
<th>Lymphocytes [%]</th>
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<tr>
<td>51</td>
<td>before colchicine</td>
<td>acute attack</td>
<td>36.3</td>
<td>28</td>
<td>62</td>
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<tr>
<td>51</td>
<td>after colchicine</td>
<td>no attacks</td>
<td>29.6</td>
<td>78</td>
<td>20</td>
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<td>before colchicine</td>
<td>acute attack</td>
<td>90.8</td>
<td>63</td>
<td>28</td>
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<tr>
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<td>after colchicine</td>
<td>no attacks</td>
<td>5.2</td>
<td>89</td>
<td>8</td>
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<tr>
<td>53</td>
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<td>acute attack</td>
<td>58.8</td>
<td>49</td>
<td>30</td>
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<td>53</td>
<td>after colchicine</td>
<td>no attacks</td>
<td>38.9</td>
<td>59</td>
<td>26</td>
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<td>Controls</td>
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<td>&lt; 5.0</td>
<td>39-77</td>
<td>22-51</td>
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### Table 3

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<th>Method</th>
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<th>2</th>
<th>3</th>
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<tr>
<td>Rule of threads</td>
<td>1.06 ± 0.24</td>
<td>1.24 ± 0.44</td>
<td>1.77 ± 0.81</td>
<td>2.10 ± 1.05</td>
</tr>
<tr>
<td>Rule of thirds</td>
<td>1.13 ± 0.34</td>
<td>1.60 ± 0.52</td>
<td>2.57 ± 0.86</td>
<td>3.21 ± 1.07</td>
</tr>
<tr>
<td>Circularity index</td>
<td>1.49 ± 0.32</td>
<td>2.08 ± 0.50</td>
<td>3.75 ± 0.94</td>
<td>4.07 ± 1.25</td>
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Table 4

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<th>Method</th>
<th>Scale of measurement</th>
<th>Explained variance [%]</th>
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<td>Phenotype-genotype association</td>
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<td>Rule of threads</td>
<td>nominal</td>
<td>16.9%</td>
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<tr>
<td>Rule of thirds</td>
<td>nominal</td>
<td>39.8%</td>
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<tr>
<td>Circularity index</td>
<td>metric</td>
<td>49.4%</td>
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Table 5

<table>
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<tr>
<th>No. of individuals</th>
<th>No. of wild type LBR alleles (and type of mutation)</th>
<th>Mean lobulation index</th>
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<tr>
<td></td>
<td></td>
<td>Rule of threads</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2.10±1.05</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>1.77±0.81</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>1.77±0.81</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>1 (null mutation)</td>
<td>1.13±0.34</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>1 (splice mutation)</td>
<td>1.34±0.48</td>
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<tr>
<td>4</td>
<td>1 (null mutation)</td>
<td>1.13±0.34</td>
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<tr>
<td>p</td>
<td></td>
<td>&lt;0.001</td>
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<tr>
<td>1</td>
<td>0 (regulatory mutation)</td>
<td>1.12±0.33</td>
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<tr>
<td>1</td>
<td>0 (splice mutation)</td>
<td>1.00±0.00</td>
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<tr>
<td>p</td>
<td></td>
<td>n.s.</td>
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Table 6

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<tr>
<th>Colchicine Dosage [µl]</th>
<th>Time [hours]</th>
<th>Mean lobulation index (p value)</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td>0 µl</td>
<td>10 h</td>
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<tr>
<td>12 µl</td>
<td>10 h</td>
<td>1.48 (n.s.)</td>
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<tr>
<td>12 µl</td>
<td>12 h</td>
<td>1.48 (n.s.)</td>
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### Table 1
Characterization of three patients with Familial Mediterranean Fever (FMF) according to the type of mutation in the MEFV-gene and colchicine treatment.

### Table 2
Clinical and laboratory data of three patients with Familial Mediterranean Fever before and after colchicine treatment. CRP = C-reactive protein.

### Table 3
Nuclear segmentation as function of the number of wild type LBR genes calculated by three different methods (means ± standard deviations).

### Table 4
Comparison of the 3 methods used for LBR phenotype quantification and two statistical approaches for variant analysis (phenotype-genotype association, norm of reaction).

### Table 5
Significance of the LBR dose effect on the lobulation index calculated by three different methods. The LBR dose depends on the number of wild type and mutated alleles (null-, splice, and regulatory mutation). Based on the “rule of threads” and the “circulatory index” the differences between all groups are significant in terms of mean values and standard deviation (Mann-Wilcoxon U test). After application of the “rule of thirds” the difference between the homozygous individual with the splice site and the regulatory mutation is not significant. p = p-value
Table 6
Mean value of the lobulation indices after treatment of whole blood with colchicine for 10 and for 12 hours, calculated by three different methods. The decrease in the lobulation index is highly significant after application of the “rule of thirds” and the “circulatory index”, but not after applying the “rule of threads” (Mann-Wilcoxon U test).

Table 7
Mean value of the lobulation indices before and after in vivo treatment with colchicine in three patients with Familial Mediterranean Fever. Based on the “rule of thirds” and the “circularity index”, a significant decrease was found in one patient (Mann-Wilcoxon U test). p = p-value
Classification of granulocyte nuclear segmentation of an individual homozygous for the wild type LBR (lamin B receptor) gene (A), heterozygous (Pelger anomaly, B) and homozygous for the mutant LBR gene (C) according to two optical classifications, "rule of threads", "rule of thirds", and a morphometric analysis, the "circularity index".
The “norm of reaction” distributions were estimated from 50 randomly selected cells per subject and assessed through the “circularity index”.

Data from (a) 2 individuals without wild type LBR gene, (b) 9 individuals with 1 wild type LBR gene, (c) 12 individuals with 2 wild type LBR genes, and (d) 3 individuals with 3 wild type LBR genes.

*x-axis*: number of lobes; *y-axis*: percentage of cells;

\( m = \text{mean} \); \( s = \text{standard deviation} \);
The "norm of variation" is represented as a graph reflecting the subject's phenotypic mean± standard deviation. All 26 subjects of our LBR sample were included and colored according to the number of wild type LBR genes: red (0), green (1), blue (2), and violet (3). Group means are plotted as dotted lines in the corresponding color.

The phenotype measure (pheno) is "rule of thirds" (a) and "circularity index" (b).
Nonlinear least squares approach to the quantitative phenotypes as derived through the “rule of thirds” method (y-axis). The 26 phenotypes (mean ± standard deviation) are represented as squares, colored according to the number of wild type LBR genes (x-axis): red (0), green (1), blue (2), and violet (3). The amount of LBR protein as derived from 7 lymphoblastoid cell lines is included as red circles.
Correlation between the number of lobes based on the "rule of thirds" and the corresponding values calculated by the "circularity index" after measurement of 1,300 granulocyte nuclei from 26 individuals with 0 to three wild type LBR genes.