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Minimal residual disease in lymph nodes after achievement of complete remission predicts time to relapse in dogs with large B-cell lymphoma

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 remission predicts time to relapse in dogs with large B-cell lymphoma
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- 4

5 Abstract

Most dogs with large B-cell lymphoma (LBCL) that undergo chemotherapy and achieve clinical complete remission (CR) eventually relapse. However, time to relapse (TTR) is unpredictable. The aims of this prospective study were to assess the influence of post-chemotherapy lymph node (LN) infiltration by large CD21+ cells using flow cytometry (FC) on TTR, and to establish a cut-off value of prognostic significance.

Dogs with newly-diagnosed, completely staged LBCL in CR after treatment were enrolled. Minimal residual disease (MRD) analysis by FC was performed on LN aspirates. TTR was calculated between MRD and relapse.

Thirty-one dogs were enrolled: 4% had stage V disease, and DLBCL was the most common histotype (74%). Based on LN infiltration at MRD evaluation, 3 groups were created: 1) acellular samples; 2) $\leq 0.5\%$ infiltration; and 3) > 0.5%infiltration. Overall median TTR was 154 days (range, 31-1974): 22 (71%) dogs relapsed during the study period, whereas 9 (29%) dogs did not. The difference among the 3 groups was significant (p=0.042 log-rank test): median TTR was not reached for dogs with LN infiltration $\leq 0.5\%$ (range, 195-

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118 days (range, 31-232) for dogs with LN infiltration >0.5%.

- 24 These results demonstrate that MRD assessment by FC on LN aspirates in
- dogs with LBCL in clinical CR predicts TTR. LN infiltration by >0.5% large
- 26 CD21+ cells after treatment is an unfavorable prognostic factor.

27

28 Keywords

canine, end-staging, flow cytometry, lymphoma, prognosis, relapse

31 Introduction

32

In dogs, multicentric lymphoma is most frequently diagnosed, and in approximately 70% of cases it is of B-cell origin.¹⁻⁴ Large B-cell lymphoma (LBCL) is characterized by medium or large-sized cells that express CD21 by flow cytometry (FC).^{5,6}

Despite significant improvements in terms of prognosis due to better 37 treatments for LBCL, most dogs that undergo first-line chemotherapy and 38 achieve clinical complete remission (CR) will ultimately relapse. The CHOP-39 based maintenance-free chemotherapeutic protocol including Prednisolone, 40 Cyclophosphamide, Doxorubicin +/-Vincristine. L-Asparaginase 41 is considered to be the gold standard treatment for dogs with LBCL.7-10 42 According to the published literature, CR rate and median first remission 43 duration range between 80-85% and 87-330 days, respectively.^{8,11-17} In dogs 44 with multicentric lymphoma the measurement of peripheral lymph nodes 45 (LNs) size after chemotherapy is used to document treatment response.¹⁸ 46 According to the Veterinary Cooperative Oncology Group (VCOG) guidelines, 47 clinical CR is defined as the regression of all affected peripheral LNs to a size 48 considered normal by physical examination using LN palpation and calipers.¹⁸ 49 Following clinical remission, the residual population of tumor cells can be 50 referred to as the minimal residual disease (MRD), which is implicated as the 51 source of tumor relapse. Therefore, measurement of MRD can provide 52

information regarding the presence of neoplastic cells even in the clinical CRphase.

Several techniques, including FC and polymerase chain reaction amplification 55 of antigen receptor genes (PARR), have been used to detect MRD in LN, 56 peripheral blood (PB) and bone marrow (BM) samples in dogs with LBCL.^{19,20} 57 Molecular techniques for detection of MRD in PB samples during or following 58 treatment can be used as an objective parameter for the determination of 59 treatment efficacy and to select dogs that might benefit from consolidation 60 chemotherapy despite clinical CR.²¹⁻²⁶ However, the universal primers 61 previously designed for PARR showed a low sensitivity for MRD evaluation.¹⁹ 62 In the current study FC was tested as an alternative method for detection of 63 MRD in the routine clinical practice. 64

65

The first aim of this prospective study was to assess the influence on time to relapse (TTR) of LN infiltration by large CD21+ cells quantified by FC in dogs with LBCL in clinical CR after treatment. The second aim was to establish a cut-off value of prognostic significance. It was hypothesized that the detection and quantification of a certain amount of neoplastic cells using FC on LNs samples at the time of MRD monitoring could help predicting early relapse.

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74 Material and methods

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76 Patient selection and inclusion criteria

Client-owned dogs with newly-diagnosed, previously untreated multicentric 77 LBCL were prospectively enrolled. To be eligible for recruitment dogs were 78 required to undergo a complete initial staging work-up (T0), consisting of 79 history and physical examination, complete blood cell count with differential, 80 serum biochemistry profile, thoracic radiographs, abdominal ultrasound, 81 cytological evaluation of liver and spleen regardless of their sonographic 82 appearance, FC on LN, PB and BM. Dogs were required to be diagnosed 83 with LBCL based on the presence of large-sized CD21+ cells using cytology 84 and FC. Dogs also underwent surgical removal of a peripheral LN to obtain a 85 histopathological diagnosis. Only dogs with a histopathological diagnosis of 86 Diffuse Large B-cell Lymphoma (DLBCL) or Marginal Zone Lymhpoma (MZL) 87 were enrolled in the study. All dogs received a CHOP-based dose-intense 88 chemotherapy protocol with an autologous vaccine.²⁷ Dogs in clinical CR 89 following treatment based on clinical, radiological, ultrasonographic and 90 cytological investigations were the subject of the analysis and underwent 91 MRD assessment using FC on post-chemotherapy LNs (T1). 92

93 Written informed consent was obtained from all owners.

94

95 Evaluation of MRD by FC

MRD evaluation by FC was performed on LN aspirates, PB and BM samples
2-4 weeks after the completion of the treatment protocol if dogs were
documented to be in clinical CR. Multiple peripheral LNs were aspirated; in
case of non-palpable nodes, ultrasound was used as a guide.

LN samples were collected into tubes containing 1 ml of RPMI 1640. PB and BM samples were collected in K3-EDTA tubes. For each matrix, a cytological specimen obtained from the same aspirate was also prepared.

Samples were delivered to the laboratory and processed as previously
 described within 24 hours from sampling.¹³

The cellularity of all matrices was tested with an automated haematology analyser (Sysmex XT-2000iV, Sysmex, Kobe, Japan). According to internal standard procedures, at least 1×10^3 nucleated cells/µl were considered necessary for the evaluation of residual disease in the LN samples (which means a total of 1×10^6 nucleated cells in the sample), otherwise the sample was reported as "acellular" and not further processed.

¹¹¹ Two tubes for FC were prepared for each matrix: one tube with unstained ¹¹² cells served as negative control. In the second tube, cells were labelled with ¹¹³ anti-CD45-FITC (clone YKIX716.13, AbD Serotec, Oxford, UK) and anti-¹¹⁴ CD21-AF647 (clone CA2.1D6, AbD Serotec) antibodies. After incubation with ¹¹⁵ antibodies, PB and BM samples underwent RBC lysis by means of a solution ¹¹⁶ containing 8% ammonium chloride. Tubes were finally washed and re-¹¹⁷ suspended in 500µl of PBS and acquired with a BD FACScalibur flow

cytometer (Becton Dickinson, San Josè, CA). For each tube, 10,000 118 nucleated cells were acquired. Analyses were performed with the specific 119 software CellQuest (Becton Dickinson). A first gate was set in the 120 morphological scattergram to exclude debris and platelets, and a second one 121 to include only CD45-positive cells. For each matrix the residual disease was 122 quantified based on the percentage of large-sized CD21-positive cells out of 123 the total CD45-positive cells (Figure 1). As cut-off value, the mean FSC value 124 125 of the neutrophil population (ranging between 350 and 400 based on our experimental condition) was used. 126

127

128 Follow-up

Dogs had to undergo follow-up evaluations to assess the remission status 129 consisting of physical examination, peripheral LNs size measurement and 130 cytological evaluation every 4 weeks during the first year, and every other 131 month thereafter. Relapse was defined as the clinical reappearance and 132 cytological evidence of lymphoma with or without FC confirmation in any 133 anatomical site in dogs having experienced CR. Once relapse was confirmed, 134 a complete restaging work-up was undertaken, and a second round of 135 chemotherapy was offered. 136

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138 Statistical analysis

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140 TTR was calculated from the assessment of MRD to relapse. Dogs lost to 141 follow-up, dead for lymphoma-unrelated causes before relapse, or those still 142 in CR at the end of the study, were censored for TTR analysis.

In order to identify the best promising cut-off to discriminate 2 prognostic groups based on LN infiltration at MRD assessment, Kaplan-Meier curves were drawn and visually inspected. The following values were arbitrarily selected and checked to this aim: 0.5%, 1.0%, 3.0% and 5.0%.

Based on data distribution, only the 0.5% cut-off was tested on PB and BMsamples.

Once the best cut-off was identified, a Cox's proportional hazard regression 149 analysis was performed, to assess the possible association of the following 150 variables with TTR: breed (purebred or crossbred), sex (male or female), age 151 (< or \ge 10 years), body weight (< or \ge 10 kg), initial stage (I to V according to 152 WHO), substage (a or b), extranodal site involvement (yes or no), anemia 153 (hematocrit < 35%, yes or no), thrombocytopenia (platelets <200,000/ μ L, yes 154 or no), serum LDH activity (< or \geq 300 IU/L) histotype (DLBCL, MZL), LN 155 group (acellular, ≤0.5% infiltrated, >0.5% infiltrated), residual disease in PB 156 (%) and BM (%). For categorical variables, Kaplan Meier curves were drawn 157 and compared with log-rank test to assess possible variations in median TTR. 158 All statistical analyses were performed using a standard statistical software 159 (SPSS 20.0 for Windows). Significance was set at $p \le 0.05$ for all tests. 160

161

162

163 **Results**

164 *Demographics*

Thirty-one dogs met the study inclusion criteria and were enrolled. There 165 were 7 (22.6%) crossbreed, and the remaining 24 (77.4%) represented 20 166 different breeds. Among the purebred dogs, there were 3 (9.7%) German 167 shepherds, 2 (6.4%) Rottweilers, 2 (6.4%) Poodles, and one (3.2%) each of 168 the following: Dachshund, Beagle, English bulldog, Shih-tzu, Dogo Argentino, 169 Doberman, Basset hound, Border collie, Shar Pei, Bernese Mountain dog, 170 Boxer, German shorthaired pointer, Akita Inu, French bulldog, American 171 staffordshire, Pomeranian, West Highland White Terrier. 172

Eighteen (58.1%) dogs were males (4 castrated) and 13 (41.9%) were females (11 spayed). The median age at diagnosis was 7 years (range, 4-12 years), whereas the median weight was 27.3 kg (range, 3.9-59.7 kg).

176

177 Clinico-pathological features

Two (6.5%) dogs were anemic, and 7 (22.6%) were thrombocytopenic. LDH activity was increased in 7 (23.3%) dogs (\geq 300 IU/L) out of 30 dogs tested, whereas serum ionized calcium was within normal limits in all cases.

Four (12.9%) dogs had stage III (substage a) disease, 14 (45.2%) had stage
IV (substage a) disease, and 13 (41.9%) had stage V disease (n=8 substage

a, and n=5 substage b). In 2 (6.5%) cases, extranodal (pulmonary) sites were
involved.

185 There were 23 (74.2%) DLBCL and 8 (25.8%) MZL.

- 186
- 187 MRD assessment on LN, PB and BM

At the time of MRD assessment, all dogs were in clinical CR. A LN aspirate was obtained in all of them and submitted for FC analysis. The aspirates obtained from 13 (41.9%) dogs were acellular. Among the 18 processed samples, median infiltration by large CD21+ cells was 1.3% (mean $5.7\pm13.0\%$; min-max 0.3-53.5%). In particular, it was $\leq 0.5\%$ in 3 (9.7%) cases, $\leq 1.0\%$ in 7 (22.6%), $\leq 3.0\%$ in 13 (41.9%), and $\leq 5.0\%$ in 15 (48.4%) dogs.

PB was tested for MRD by FC in all 31 cases. Median infiltration was 0.3% (mean 0.41 \pm 0.34%; min-max 0.1-1.5%). In particular, it was \leq 0.5% in 21 (67.7%) dogs.

BM was tested for MRD by FC in 25 (80.6%) cases. Median infiltration was 0.3% (mean 0.51 \pm 0.49%; min-max 0.1-1.9%). In particular, it was \leq 0.5% in 16 (64%) dogs.

201

202 Outcome and prognostic groups based on LN infiltration

203 Overall median TTR was 154 days (range 31-1974 days). Twenty-two (71%) 204 dogs relapsed during the study period, whereas 9 (29%) were censored for

205 TTR (8 were still alive and in CR at data analysis closure and 1 died from 206 unrelated causes still being in CR for lymphoma after 61 days).

Among the 4 cut-offs tested for LN infiltration, only 0.5% discriminated among prognostic groups (Figure 2). Thus, three groups were created for statistical analysis, based on LN infiltration at MRD assessment: 1) acellular LN sample; 2) \leq 0.5% infiltration; and 3) >0.5% infiltration. Conversely, no discriminating cut-off was identified for PB and BM samples.

The three LN groups significantly differed for median TTR according to logrank test (p=0.042), but not according to Cox analysis (p=0.067). Nevertheless, significant results were obtained with Cox analysis when LN groups 1 and 2 were coupled together and compared with samples >0.5% infiltration (p=0.030, Figure 3). None of the remaining variables significantly influenced TTR by either test. Median TTR and p-values for all variables are listed in Table 1.

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220

221 **Discussion**

222

223 Monitoring MRD is crucial for dogs with LBCL undergoing treatment. Relapse 224 is the major cause of treatment failure and decrease in survival rate, and it 225 emerges from the outgrowth of residual treatment-resistant neoplastic cells.²⁸ 226 While diagnosing an overt relapse is quite straightforward, recognizing the

presence of residual neoplastic cells if peripheral LNs are not enlarged is challenging. The current prospective study shows that MRD assessment by FC on LN samples in dogs with LBCL in clinical CR may predict TTR, thereby representing a useful tool for disease monitoring after therapy completion. In detail, dogs with >0.5% LN infiltration were more likely to relapse despite the clinical CR.

According to the VCOG guidelines, all dogs in the present study were in 233 clinical CR at the end of treatment¹⁸, yet 50% of them had detectable MRD 234 based on FC, ultimately leading to early relapse. Thus, FC may play a role 235 not only in the initial diagnostic work up^{5,6,13,29,30}, but also in post-treatment 236 surveillance. In fact, cytology and even imaging may occasionally detect 237 clinically occult relapse, but the benefit of these techniques has not been 238 demonstrated in LBCL. Whether the detection of relapse ahead of clinical 239 symptoms translates into a clinical benefit was not investigated in the current 240 study, therefore it is currently unknown whether early MRD-based 241 intervention can affect outcome. Nevertheless, these results allow at least the 242 question to be addressed in clinical trials, by including MRD assessment at 243 the end of treatment to document the presence or absence of subclinical 244 disease. 245

246

The second aim of this study was to establish a cut-off value of prognostic significance for TTR. The identification of a MRD cut-off value might

represent an objective and repeatable tool for risk assessment, by identifyingthose dogs that are at risk of relapse, yet clinically in remission phase.

In the current study 22 (71%) dogs relapsed during the study at different time points (31-1974 days). By applying a cut-off value of 0.5%, two prognostic groups were identified: an early relapse group (median TTR 118 days) and a late relapse group (median TTR not reached).

Because dogs with MRD >0.5% at the end of treatment had the shortest TTR, it was hypothesized that MRD could not only represent the best indicator for the risk of relapse, but also a substantial aid to decide the interval at which dogs should be monitored, possibly leading to treatment intensification.

The period between clinical remission and clinical relapse was different in dogs in the early relapse group. The clinical relapse occurred after a median interval of 118 days of MRD assessment. This period was even shorter in some dogs, thereby indicating that the evolution of disease is not completely similar in all dogs despite being categorized in the same risk group.

Based on the above, we strongly recommend to assess the clinical remission status on a biweekly basis if MRD is above the defined threshold, as a followup interval of 2 weeks allows predicting the clinical relapse events before clinical manifestations.

268

Conversely, dogs with MRD levels ≤0.5% or those in which LN samples were
 acellular were mostly associated with a good outcome. While the threshold of

 $\leq 0.5\%$ is intuitive, one may argue the meaning of acellular samples.

In the current study, at least 1×10^6 intact cells ($\geq 1 \times 10^3$ nucleated cells/µl) 272 were considered necessary for the evaluation of MRD by FC. This threshold 273 was arbitrarily set, since this amount of cells is required for the application of 274 a minimal immunophenotyping panel according to internal standard 275 procedures. Indeed, working dilutions of the antibodies have been 276 established for a concentration of 500,000 cells/tubes. While the reduced 277 panel of antibodies used here may be adapted to less cellular samples, thus 278 possibly reducing the percentage of "acellular" cases, it must be noted that 279 statistically the acellular samples tended to overlap with those with <0.5% 280 infiltration in terms of outcome. The most likely explanation for this finding is 281 that dogs achieving CR after treatment undergo LNs atrophy, as previously 282 documented.¹⁹ Fine-needle aspirates obtained from one or more peripheral 283 LNs were always evaluated by means of cytology prior to FC analysis, to 284 ensure that a LN was actually sampled. Despite having performed 285 ultrasound-guided fine-needle aspiration of multiple peripheral non-palpable 286 LNs and having obtained good cytological smears (data not shown), the 287 cellularity of atrophic LNs was inadequate for flow cytometric analysis in 13 288 dogs, yielding acellular samples. Nevertheless, dogs with acellular samples 289 at the end of treatment experienced a long TTR. Based on the above, 290 acellular samples by FC suggest that residual neoplastic cells are virtually 291 absent in the LNs, ultimately being associated to a good prognosis. 292

293

294 This study has some limitations.

While cytology and FC are commonly used for diagnosing canine lymphoma, 295 histopathology is still not routinely performed. Despite the group enrolled in 296 the current study was homogeneous as far as cytological and FC features, 297 histopathology showed that there were 23 aggressive (DLBCL) and 8 indolent 298 lymphomas (MZL). Further development and validation of MRD techniques 299 are required to confirm that FC detection of residual neoplastic cells is 300 feasible and prognostic among all lymphoma subtypes. Also, dogs underwent 301 one single MRD testing 2-4 weeks after the end of treatment. This time point 302 was arbitrarily selected. It is possible that MRD assessment at different time 303 points or incorporated in serial monitoring could add value for clinical 304 management. 305

MRD testing itself has some technical and practical limitations, as a 306 significant number of MRD-negative dogs still relapse. First, while the 307 cellularity of samples is crucial for FC analysis, the minimal amount of 308 neoplastic cells that permits sample processing has not been clearly 309 identified. Second, clonality of putative neoplastic cells cannot be tested by 310 FC. We assessed the percentage of large CD21+ cells in LNs after treatment 311 because these cells have the same FC morphological and immunophenotypic 312 features of neoplastic cells at diagnosis. Still, medium or large reactive B-313 cells may have been included in the count, thereby falsely increasing MRD 314

values. Finally, it would be interesting to evaluate if LN aspirates from different sites may influence the percentage of infiltration thus causing migration from one relapse group to another. All these aspects could be better elucidated in specific studies aimed to create standardized recommendation for the assessment of MRD via FC in dogs with LBCL.

320

In conclusion, the present study indicates that MRD assessment by FC on LN 321 aspirates is a useful tool for assessing the presence of subclinical disease in 322 dogs with LBCL treated with chemo-immunotherapy. Relapse occurrence 323 could be efficiently predicted through FC prior to clinical relapse diagnosis, 324 and the value >0.5% was associated with early recurrence. Further trials 325 randomizing dogs according to their MRD-status are required to assess 326 whether MRD-guided management of dogs with LBCL will ultimately lead to 327 improved outcome and personalized care. 328

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331 Conflict of interest

None of the authors of this paper has a financial or personal relationship with
other people or organizations that could inappropriately influence or bias the
content of the paper.

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437

438 Table 1: Time To Relapse (TTR) in 31 dogs with large B-cell lymphoma

439 (LBCL), according to different variables

Variables (number of	Median TTR in	p-value	
dogs)	days (range)	Univariate	Log-rank test
		analysis	
Breed		0.542	0.538
- purebred (24)	154 (31-911)		
- crossbred (7)	144 (42-1974)		
Sex		0.317	0.311
- male (18)	126 (42-449)		
- female (13)	157 (31-1974)		
Age		0.317	0.339
- <10 years (19)	126 (31-911)		
- ≥10 years (12)	175 (75-1974)		
Body weight		0.378	0.370
- <10 kg (5)	449 (31-449)		
- ≥10 kg (26)	154 (42-1974)		
Stage		0.530	0.509
- III (4)	154 (63-1974)		
- IV (14)	145 (42-449)		
- V (13)	154 (31-911)		

Substage		0.437	0.431
- a (26)	154 (31-1974)		
- b (5)	191 (42-195)		
Extranodal site		0.607	0.601
involvement			
- yes (2)	69 (69;429)		
- no (29)	154 (31-1974)		
Anemia		0.537	0.531
- yes (2)	42 (42;195)		
- no (29)	154 (31-1974)		
Thrombocytopenia		0.674	0.672
- yes (7)	157 (42-1206)		
- no (24)	154 (34-1974)		
Serum LDH activity		0.950	0.950
- normal (23)	154 (42-1974)		
- increased (7)	126 (31-429)		
Histotype		0.652	0.650
- DLBCL (23)	154 (31-1974)		
- MZL (8)	191 (61-429)		
Residual disease in		0.067	0.042
lymph nodes			
- ≤0.5% (3)	Not reached		

- >0.5% (15)	(195-429)		
- Acellular sample	118 (31-232)		
(13)	164 (63-1974)		
Residual disease in		0.608	
peripheral blood (%)			
(31)			
Residual disease in		0.392	
bone marrow (%) (25)			

440

441 **Figure 1**

Flow cytometric scattergrams showing Minimal Residual Disease (MRD) 442 evaluation in 3 dogs with Large B-Cell Lymphoma (LBCL). Left column: a first 443 gate in a morphological scattergram was set to exclude platelet and debris 444 (R1). Central column: only R1 events are shown; a second gate was set to 445 include only CD45-positive events. Right column: only events included in both 446 R1 and R2 are shown; MRD was calculated as the percentage of cells in the 447 upper right quadrant (large-sized CD21-positive cells). The three rows show 3 448 cases with different MRD levels. 449

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451

452 Figure 2

Kaplan-Meier curves showing Time To Relapse (TTR) in 31 dogs with large 453 B-cell lymphoma (LBCL) according to flow cytometric (FC) results on post-454 chemotherapy lymph node (LN) aspirates, peripheral blood (PB) and bone 455 marrow (BM) samples. Dashed line: large CD21+ cells \leq the cut-off. 456 Continuous line: large CD21+ cells > the cut-off. Dotted line: acellular 457 samples. A-D: cases were classified according to percentage of residual 458 disease in LN aspirates. A: the cut-off was set at 0.5%. B: the cut-off was set 459 at 1.0%. C: the cut-off was set at 3.0%. D: the cut-off was set at 5.0%. E: 460 cases were classified according to the percentage of residual disease in PB 461 samples; the cut-off was set at 0.5%. F: cases were classified according to 462 the percentage of residual disease in BM samples; the cut-off was set at 463 0.5%. 464

465

466 **Figure 3**

Kaplan-Meier curves showing Time To Relapse (TTR) in 31 dogs with large B-cell lymphoma (LBCL) according to flow cytometric (FC) results on postchemotherapy lymph node (LN) aspirates. Continuous line: large CD21+ cells > 0.5%. Dotted line: large CD21+ cells \leq 0.5% and acellular samples. The difference between the two groups was statistically significant (p=0.030).

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