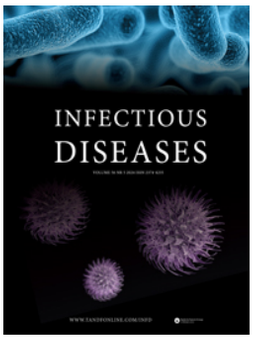

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Ondrej Bouska, Vladimira Koudelakova, Sona Gurska, Katerina Kubanova, Rastislav Slavkovsky, Hana Jaworek, Jana Vrbkova, Petr Dzubak & Marian Hajduch

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RESEARCH ARTICLE

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Pooling of samples to optimise SARS-CoV-2 detection in nasopharyngeal swabs and gargle lavage self-samples for covid-19 diagnostics and surveillance

Ondrej Bouska[a], Vladimira Koudelakova[a,b], Sona Gurska[a], Katerina Kubanova[a], Rastislav Slavkovsky[a], Hana Jaworek[a,b], Jana Vrbkova[a], Petr Dzubak[a] and Marian Hajduch[a,b]

[a]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic; [b]Laboratory of Experimental Medicine, University Hospital Olomouc, Olomouc, Czech Republic

ABSTRACT

Background: Testing of pooled samples is an effective strategy for increasing testing capacity while saving resources and time. This study aimed to validate pooled testing and gather real-life data on its use for Covid-19 surveillance with a gargle lavage (GL) self-sampling strategy.

Methods: Two-stage pooled testing with pools of 6 and 12 samples was used for preventive testing of an asymptomatic population and Covid-19 surveillance in Czech schools. Both GL and nasopharyngeal swabs were used for sampling.

Results: In total, 61,111 samples were tested. The use of pooled testing for large-scale Covid-19 surveillance reduced consumable costs by almost 75% and increased testing capacity up to 3.8-fold compared to standard methods. RT-PCR experiments revealed a minimal loss of sensitivity (0–2.2%) when using pooled samples, enabling the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genes with Ct values >35. The minor loss of sensitivity was counterbalanced by a significantly increased throughput and the ability to substantially increase testing frequencies.

Conclusions: Pooled testing is considerably more cost-effective and less time-consuming than standard testing for large-scale Covid-19 surveillance even when the prevalence of SARS-CoV-2 is fluctuating. Gargle lavage self-sampling is a non-invasive technique suitable for sample collection without a healthcare worker's assistance.

KEYWORDS
Pooled testing
self-sampling
gargle lavage
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CONTACT
Vladimira Koudelakova
vladimira.koudelakova@upol.cz
Marian Hajduch
marian.hajduch@upol.cz
Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and Laboratory of Experimental Medicine, University Hospital Olomouc, Hnevotinska 5, 775 15 Olomouc, Czech Republic

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Background

The persisting Covid-19 pandemic caused by highly transmissible SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) has affected nearly the entire population of the world and all of its socio-economic spheres [1]. According to the World Health Organization's weekly reports, there have been over 762 million confirmed Covid-19 cases and 6.8 million deaths globally since the initial outbreak in late December 2019 [2]. The world has faced several Covid-19 waves over the last three years, resulting in rapidly changing local interventions to limit viral spread, including social distancing, school closures and interventions targeting healthcare systems (HCSs). The cyclical spread of SARS-CoV-2 has been influenced by seasonal, social and political factors [3,4] as well as the emergence of new SARS-CoV-2 variants [5–7]. In addition, the pandemic's dynamic and rapidly changing nature has necessitated the development of reliable sampling strategies that can be implemented in different settings and pandemic phases. It has also placed unprecedented and rapidly changing demands on HCS, leading to massive episodic supply shortages and challenging work conditions for healthcare employees. The gold standard for SARS-CoV-2 testing is nasopharyngeal swab (NPS) sampling followed by RNA isolation and RT-PCR detection [8,9]. However, given the urgent requirement for rapid and reliable SARS-CoV-2 detection, there was a clear need to develop and evaluate simplified laboratory protocols and methods for SARS-CoV-2 detection to reduce burdens on HCS. Consequently, several studies have evaluated testing procedures using alternative types of biological material, extraction-free SARS-CoV-2 detection methods, rapid antigen detection tests (RADTs), serological assays, self-sampling and pooled sample testing [10–14].

This study evaluates the implementation of gargle lavage (GL) self-sampling and sample pooling in large-scale testing in two different settings. Sample pooling is a well-established diagnostic strategy for large-scale testing that was first applied by Dorfman [15]. Several different pooling approaches have since been developed and used to screen for various diseases, including Covid-19 [15–18]. In addition, several different types of clinical samples have been used for SARS-CoV-2 screening instead of the gold standard NPS samples, including oropharyngeal swabs, saliva, exhaled breath condensates and self-collected GL samples [8, 14, 19]. Our goal was to prove that adopting a time- and resource-saving strategy that combines self-sampling and sample pooling can provide an immediate increase in testing capacity in real-life settings while reducing testing costs, having a minimal impact on sensitivity, and placing few additional demands on laboratory staff.

Materials and methods

Validation study

The detection of SARS-CoV-2 in pools containing 6 and 12 samples was validated using 45 SARS-CoV-2 positive GL samples. These samples were selected based on prior testing using the SARS-CoV-2 Nucleic Acid Detection Kit (PCR Fluorescent Probe Method; Zybio Inc., Chongqing Municipality, China). The outcomes were then used to categorise the 45 positive samples into three groups based on their cycle thresholds (Ct), requiring at least two out of three targeted SARS-CoV-2 genes to fulfil the group-defining criteria. The Ct value served as an indirect gauge of viral load. Fifteen samples were classified as highly positive ($25 \leq Ct < 30$), 15 as moderately positive ($30 \leq Ct < 35$) and the remaining 15 as weakly positive ($Ct \geq 35$). The pool of SARS-CoV-2 negative GL samples was utilised to dilute the positive samples in the appropriate ratio. Samples were pooled in ratios of 1:6 and 1:12. The objective was to determine the detectability of SARS-CoV-2 across various levels of viral load.

RNA isolated using the Nucleic Acid Extraction Kit on automatic nucleic acid extractor Zybio EXM6000 (both Zybio Inc., Chongqing Municipality, China) was processed with three commercial RT-PCR assays to compare their performance and usefulness for pool testing: the Novel Coronavirus (2019-nCoV) Real-time Multiplex RT-PCR Kit (Liferiver, La Jolla, CA), henceforth referred to as 'Liferiver RT-PCR', the 3DMed 2019-nCoV RT-qPCR Detection Kit (3D Biomedicine Science & Technology Co., Ltd., Beijing, China), henceforth referred to as '3DMed RT-PCR', and the SARS-CoV-2 Nucleic Acid Detection Kit (PCR Fluorescent Probe Method; Zybio Inc., Chongqing Municipality, China), henceforth referred to as Zybio RT-PCR. All RT-PCR assays were used according to the manufacturer's instructions and all pools of 6 and 12 samples were tested within the same run.

Clinical study design

This study is a retrospective cross-sectional evaluation of time efficiency, cost efficiency and performance of SARS-CoV-2 testing by sample pooling compared to standard testing in single reactions in an asymptomatic population. The study was performed at the Faculty of

Medicine and Dentistry at Palacky University and the University Hospital in Olomouc between August 2021 and February 2022. The study was performed in compliance with the Helsinki Declaration according to a study ethics proposal approved by the Ethics Committee of the Faculty of Medicine and Dentistry at Palacky University and the University Hospital in Olomouc (protocol no. 162/20).

Sample collection

The first part of this study (arm A), conducted between 2 August 2021 and 12 October 2021, involved routine screening of an asymptomatic population. In total, 32,598 clinical samples were collected at the Covid-19 sampling points of the University Hospital in Olomouc. NPSs or GL samples were collected, depending on the patients' preferences: 27,265 NPS samples collected by clinicians using an ESwab collection system (Copan, Mantua, Italy) and 5333 GL self-samples collected using GARGTEST sampling kits (IntellMed, Olomouc, Czech Republic) under medical supervision.

In the second part of the study (arm B), samples from 5853 students and employees at 10 primary and secondary schools in the Olomouc region were tested weekly. Weekly (Monday or Tuesday) self-sampling of students/school employees between the 13 December 2021 and the 14 February 2022 was a part of the Czech Covid-19 national screening programme in schools. In total, 28,602 GL self-samples were collected using the GARGTEST sampling kit.

Each sample was barcoded, immediately delivered to the laboratory according to the manufacturer's recommendations, heat-inactivated at 65 °C for 20 min and tested for SARS-CoV-2.

Sample pooling

A two-stage Dorfman pooling strategy was used forSARS-CoV-2 detection [15]. Dorfman pooling is currently the simplest of the pooling methods. It has the advantage of a simple pooling algorithm, allowing high scalability and easy recognition of true positive samples. Only samples from positive pools are identified for retesting in separate reactions. Freedom EVO Clinical 150 and Freedom EVO Clinical 200 automatic pipettors (Tecan Group Ltd., Männedorf, Switzerland) were used for liquid handling during the pooling process. Samples were combined blindly in the order they were transported and received in the laboratory.

In brief, the pooling protocol consists of two stages. In the first stage, reserve plates holding 450 µl of heat-inactivated primary samples were prepared by pipetting robots (Figure 1). Simultaneously, pooled samples with a total volume of 200 µl each were created by mixing equal volumes of primary samples. Six-sample pools were created by mixing 33 µl aliquots of six primary samples in a well of a 96-well isolation plate, enabling the analysis of 480 primary samples per plate in total. Twelve-sample pools were created by mixing 17 µl aliquots of 12 primary samples, enabling the analysis of 1056 primary samples per plate. RNA was then isolated from each pooled sample and used as a template in the real-time reverse transcription polymerase chain reaction (RT-PCR). Then, SARS-CoV-2 positive pools were identified. In the second stage, every sample from a positive pool was retested individually, using 200 µl of the primary sample to identify true SARS-CoV-2 positive samples in the pool.

Labelled reserve plates with heat-inactivated primary samples were sealed and stored at 4 °C until the retesting of samples from positive pools. Complete testing was finished within 24 h, after which the reserve plates were liquidated. The volume of stored primary samples was sufficient for the second stage of testing and potentially another round if the results were inconclusive.

RT-PCR SARS-CoV-2 detection

To maximise efficiency and avoid resource wastage, we used two very similar nucleic acid extraction kits: the Nucleic Acid Extraction Kit and scalable RNA Viral Prep 480 (IntellMed, s.r.o., Olomouc, Czech Republic). Both RNA isolation methods were performed on automatic nucleic acid extractor Zybio EXM6000 with sample volume of 200 µl and elution volume of 50 µl according to the manufacturer's recommendations. RT-PCR was performed using SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method) according to the manufacturer's recommendations. RT-PCR results for pool reactions were evaluated according to the criteria listed in Supplementary Table 1 and individual reactions were evaluated as described previously [20].

Statistical and cost–benefit analysis

The statistical software R (version 4.1.0; R Core Team, R Foundation for Statistical Computing, http://www.r-project.org) was used for data evaluation, summarisation and graphical presentation. The non-parametric

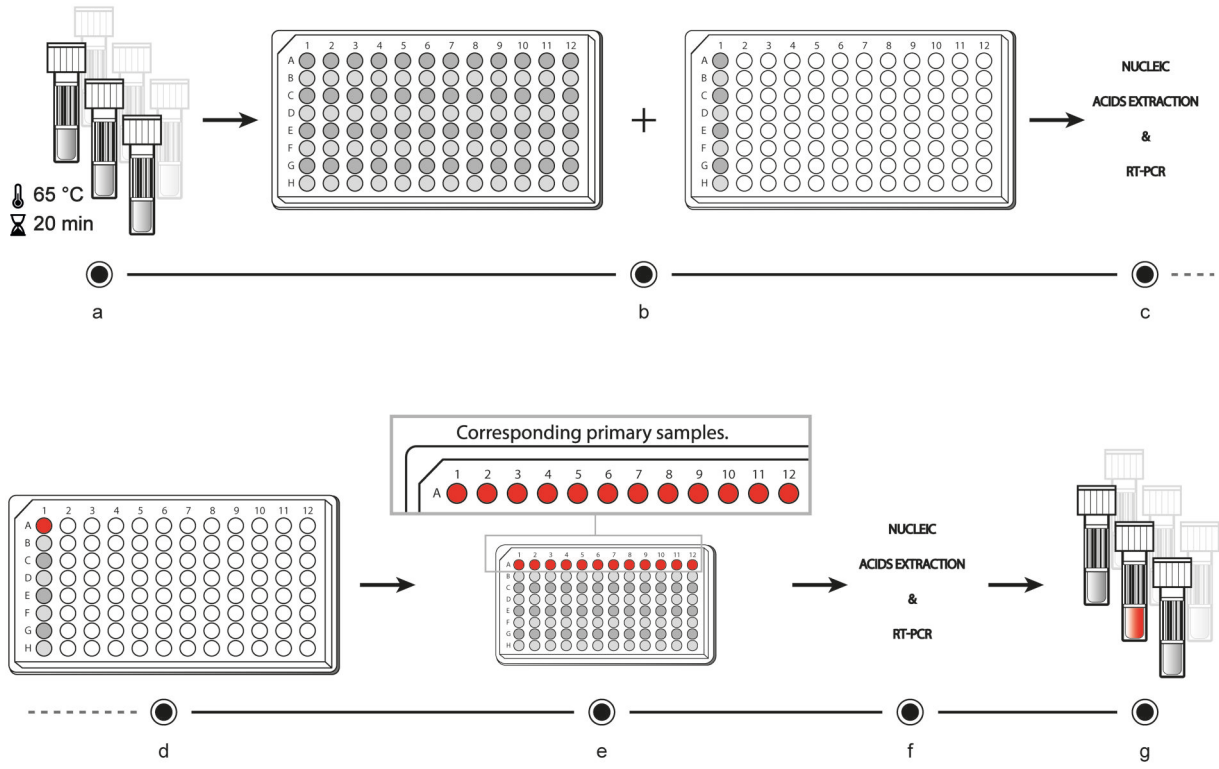

Figure 1. Schematic illustration of the pooling workflow for testing of 6- or 12-sample pools and subsequent tracing of SARS-CoV-2 positive samples: (a) sample delivery and heat inactivation, (b) reserve primary sample plate preparation and sample pooling, (c) first round of RNA extraction and RT-PCR, (d) identification of SARS-CoV-2 positive pools, (e) selection of the corresponding primary samples from reserve plates, (f) second round of RNA extraction and individual sample RT-PCR and (g) tracing of individual SARS-CoV-2 positive samples.

one-sample Wilcoxon test was used to evaluate differences between pairwise Ct values from single and pooled reactions for each pool type (6 and 12 samples) and gene (*N*, *RdRP*, *E*). Correlations between individual and pooled Ct values were evaluated using the Spearman rank correlation coefficient, which was tested against zero. Statistical significance is reported using *p* values for the relevant tests. Cost–benefit analysis was performed in software R, all details are summarised in Supplementary File 1.

Results

Validation study

To evaluate the accuracy of SARS-CoV-2 detection when using sample pooling, we performed a validation study using three different RT-PCR detection assays and 45 positive SARS-CoV-2 gargle samples. The detection rates for the Zybio, Liferiver and 3D Med RT-PCR assays were 100%, 88.9% and 73.3%for six-sample pools and 97.8%, 84.4% and 57.8% for 12-sample pools, respectively (Supplementary Table 2).

Additional analyses were performed using the SARS-CoV-2 Nucleic Acid Detection Kit (Zybio Inc., Chongqing Municipality, China) based on the above results. Analyses of paired Ct values for single and pooled samples revealed negligible Ct value shifts and losses of PCR assay sensitivity (Figure 2): the median Ct value shifts for six-sample pools were 0.37 ($p = .107$; *N* gene), −0.04 ($p = .818$; *RdRP* gene) and −0.87 ($p = .022$; *E* gene), while those for 12-sample pools were 0.79 ($p = .002$; *N* gene), 0.87 ($p = .003$; *RdRP* gene) and 0.1 ($p = .37$; *E* gene). The individual and pool Ct values (Supplementary Figure 1) for each gene were strongly correlated, with correlation coefficients of $r = 0.84$ (*N* gene), $r = 0.85$ (*RdRP* gene) and $r = 0.85$ (*E* gene) for six-sample pools ($p < .001$), and $r = 0.84$ (*N* gene), $r = 0.87$ (*RdRP* gene) and $r = 0.83$ (*E* gene) for 12-sample pools ($p < .001$). Based on these results, the SARS-CoV-2 Nucleic Acid Detection Kit (Zybio Inc., Chongqing Municipality, China) was chosen for routine SARS-CoV-2 screening using sample pooling. Zybio detected all except one 12-sample positive pools. In this pool, a sample with very low SARS-CoV-2 positivity (Ct = 38.67 (*E* gene); Ct = 35.55 (*N* gene); Ct = not detectable (*RdRP* gene)) was included.

Positivity rates

Twelve-sample pooling was used to test 32,598 samples from arm A of our study. The local SARS-CoV-2 positivity

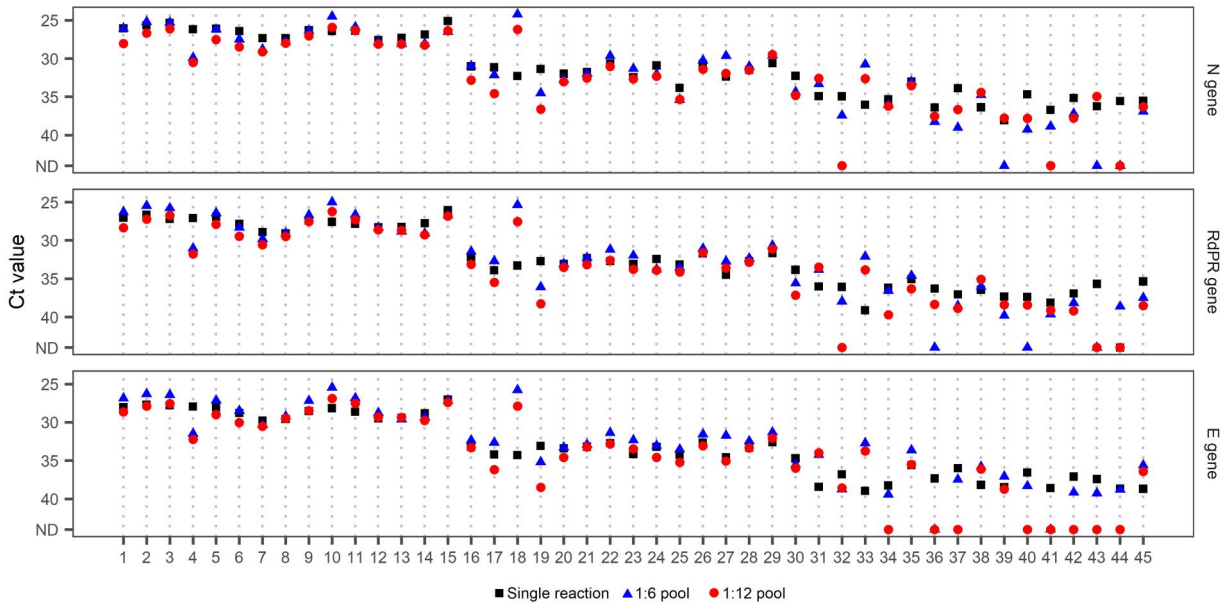

Figure 2. Results of a validation study using 45 SARS-CoV-2 positive gargle lavage samples and the Zybio RT-PCR assay. Data for single samples, six-sample pools and 12-sample pools are shown using black squares, blue triangles and red dots, respectively. *E* gene: *E* gene of SARS-CoV-2; *N* gene: *N* gene of SARS-CoV-2; *RdRP*: gene for RNA-dependent RNA polymerase of SARS-CoV-2.

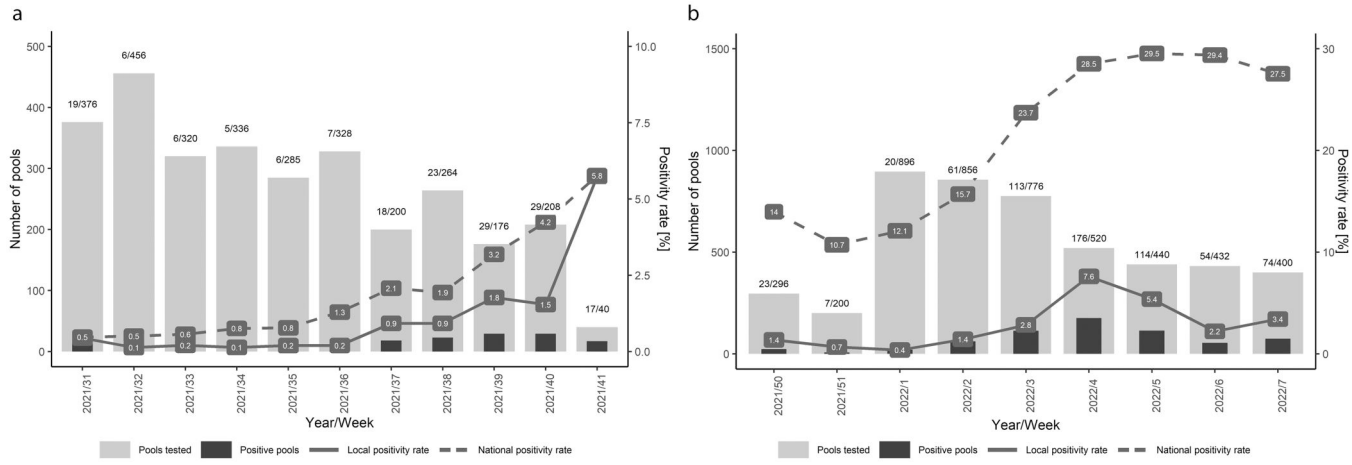

Figure 3. Statistical overview of weekly tested pools and positivity rates. (a) Routine testing of asymptomatic people from Covid-19 sampling points at the University Hospital in Olomouc (arm A). Weekly counts of tested 12-sample pools (light grey bars) and SARS-CoV-2 positive sample pools (dark grey bars) together with the proportions of positive pools and total numbers of tested pools. The continuous line represents the local weekly positivity rate in the asymptomatic population, calculated as the ratio of weekly SARS-CoV-2 positive samples to the total number of weekly samples tested at our testing centre. The overall national weekly positivity rate is shown by the dashed line. (b) Covid-19 surveillance performed at schools in the Olomouc region (arm B). Weekly counts of tested six-sample pools (light grey bars) and SARS-CoV-2 positive sample pools (dark grey bars) together with the proportions of positive pools and total numbers of tested pools. The continuous line represents the weekly positivity rate in students and school employees, calculated as the ratio of weekly SARS-CoV-2 positive samples to the total number of tests performed weekly during school Covid-19 surveillance at our testing centre. The overall national weekly positivity rate is shown by the dashed line.

rate among the asymptomatic population tested at our centre was 0.6% (0.1–5.8%, Figure 3(a)), while the average overall national positivity rate was 2% (0.5–5.8%, national data available at https://onemocneni-aktualne.mzcr.cz/covid-19). In total, 179 SARS-CoV-2 positive samples were detected in arm A (Table 1). Due to an increase in the SARS-CoV-2 positivity rate and a decrease in the number of asymptomatic patients, we switched from pooled testing to individual sample testing at the beginning of week 41 in 2021. During Covid-19 surveillance in schools (arm B), six-sample pooling was used to test 28,602 GL samples. The average positivity rate at schools was 2.7% (0.3–7.6%; Figure 3(b)), and the average overall national positivity rate was 20.5% (10.7–29.5%). In total, 762 SARS-CoV-2 positive samples were detected in arm B (Table 1).

Table 1. Summary table.

	Total	1:12 pools	Incomplete 1:12 pools[a]	1:6 pools	Incomplete 1:6 pools[a]
Number of samples tested	61,111	27,000	5509	28,356	246
Number of pools tested	7708	2250	732	4726	59
Number of positive pools	936	123	46	759	8
Number of negative pools	6831	2127	686	3967	51
PCR reactions performed[b]	14,200	3726	1098	9280	96
Number of positive samples	941	132	47	754	8
Empirical efficiency[c]	4.3	7.3	–	3.1	–
Saved tests (%)[d]	46,911 (77)	23,274 (86)	4411 (80)	19,076 (67)	150 (61)
Cost savings (%)[e]	410,271 € (75)	225,072 € (16)	–	185,199 € (34)	–

[a]Incomplete pools with less than 12 or 6 mixed samples.
[b]The data include the number of RT-PCR pool reactions as well as the number of individual reactions needed for SARS-CoV-2 positive sample tracing.
[c]Calculated as the ratio of actually performed reactions (both pool and individual) and the number of individual reactions if only single reactions were used for each collected sample.
[d]Number of individual reactions saved by pool testing compared to testing individually (RNA isolation and RT-qPCR).
[e]Cost savings compared to testing in individual reactions (including costs for consumables and salaries of three laboratory technicians and one certified healthcare professional).

Table 2. Comparison of testing costs and testing capacity in different settings.

			Proportional capacity increase	
Testing approach	Cost per sample (€)[a]	Testing capacity (daily)[b]	vs. 96-single r.	vs. 384-single r.
1:12 pool reaction[c]	1.1	4200	3.8	2.0
1:6 pool reaction[c]	1.9	3300	3.0	1.6
384-single reaction[d]	6.0	2050	1.9	–
96-single reaction	9.9	1100	–	–

[a]The final amount includes the cost of consumables and salaries (three laboratory technicians and one certified healthcare professional).
[b]The daily testing capacity was determined in a laboratory staffed by three laboratory technicians and one certified healthcare professional with two liquid handlers, two nucleic acid isolation systems and two real-time PCR cyclers.
[c]Pool reactions performed in 96-well plate format.
[d]Testing in 384-well plate format with reaction volumes reduced by 75% compared to the 96-well case.

Efficiency of pool testing

The individual empirical efficiency, defined as the number of PCR tests needed to test all samples divided by the number of actually performed tests, was 3.1 for six-sample pools and 7.3 for 12-sample pools, while the overall empirical efficiency was 4.3 (Table 1). Sample pooling also reduced overall consumable costs by 75% compared to testing in individual reactions. The throughput capacity when testing pools of 12 and 6 samples in a real laboratory was about 3300 and 4200 samples per day (Table 2), respectively. The 12-sample and six-sample pooling strategies thus increased testing capacity 3.8-fold and 3.0-fold compared to single reactions and 2.0-fold and 1.6-fold compared to single reactions using 384-well plates. Twelve- and six-sample pooling also reduced testing costs to 1.1 € and 1.9 € per sample, respectively (Table 2).

Comparison of pooled and single reactions in arm A

The paired Ct values for sample pools and individual testing from the second stage revealed Ct shifts of 3.38, 3.68 and 2.97 for the *E*, *RdRP* and *N* genes (p value $<$ .001) in cases where only one SARS-CoV-2 positive sample was present in a tested pool (Figure 4(a), Supplementary Table 3). The Ct value shifts of SARS-CoV-2 genes in pools with $\geq$2 SARS-CoV-2 positive samples (Figure 4(b), Supplementary Table 3) were 1.79, 1.33 and 0.72 for the *E*, *RdRP* and *N* genes, but paired Ct values for individual samples did not differ significantly from the 12-sample pools. A combined comparison revealed a high correlation between the Ct values for pooled and individual samples (Supplementary Figure 2) ($r(N) = 0.66$, $r(RdRP) = 0.67$, $r(E) = 0.66$; p value $<$.001). All primary samples from 6/123 positive pools (4.9%) were found to be negative when retested individually. In the remaining 117 positive pools, one positive primary sample was found in 104 cases (84.6%), and $\geq$2 SARS-CoV-2 positive primary samples were found in 13 cases (10.6%) (Supplementary Figure 3).

Comparison of pooled and single reactions in arm B

The Ct shifts observed when comparing results of six-sample pools and individual samples from the second stage, containing only one SARS-CoV-2 positive sample, were 2.82, 3.22 and 3.27 for the *E*, *RdRP* and *N* genes, respectively (p value $<$.001) (Figure 5(a), Supplementary Table 4). The median Ct value shifts in pools with $\geq$2 SARS-CoV-2 positive samples were 1.26, 1.57 and 1.88 for the *E*, *RdRP* and *N* genes, respectively (p value $<$.001) (Figure 5(b), Supplementary Table 4). A combined comparison of Ct values revealed strong correlations

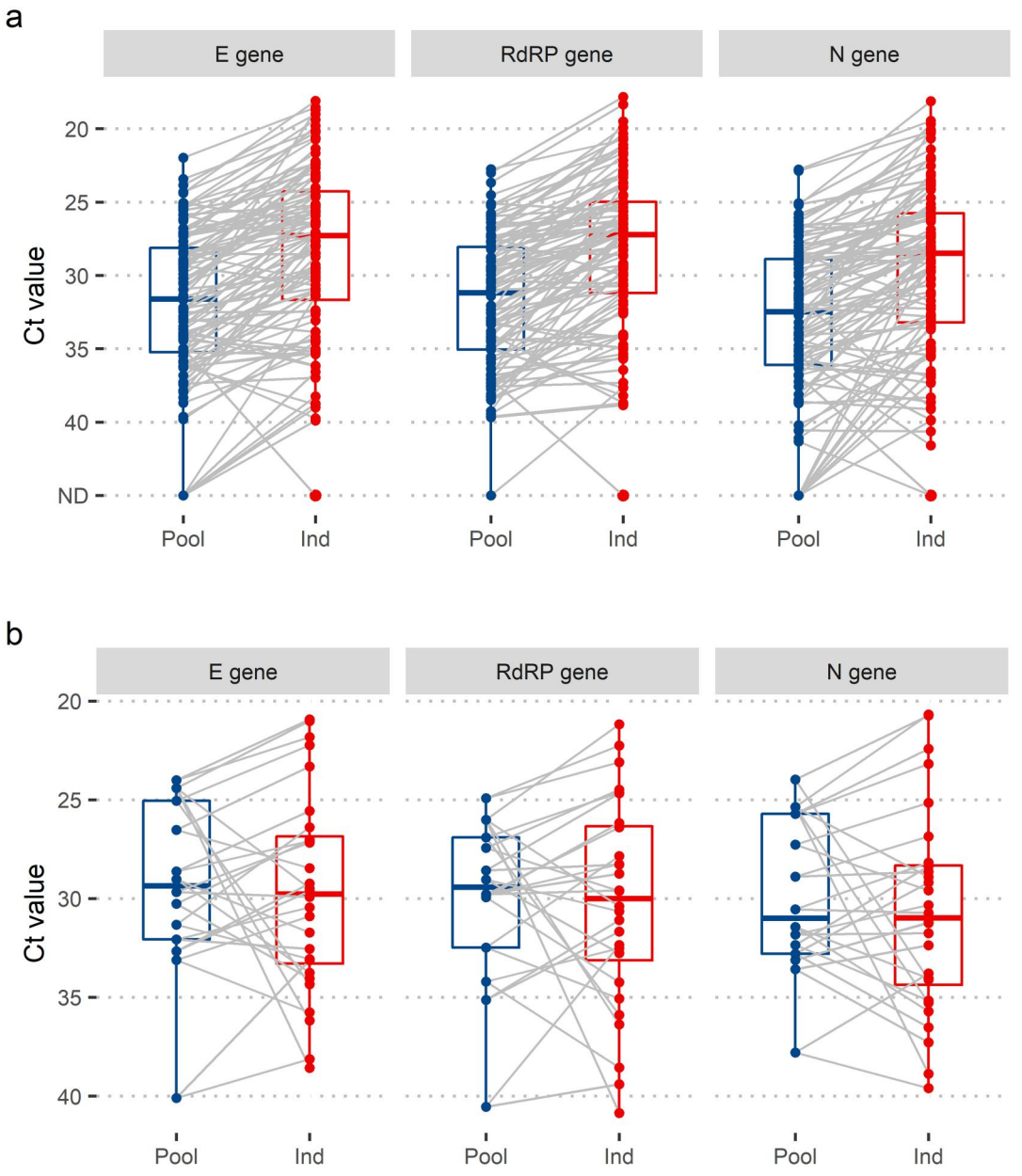

Figure 4. Comparison of paired RT-PCR results for individual samples (Ind) and 12-sample pools (Pool) in arm A for pools with (a) only one SARS-CoV-2 positive sample and (b) ≥2 SARS-CoV-2 positive samples. Incomplete pools were excluded from the final analysis of paired results. *E* gene: *E* gene of SARS-CoV-2; *N* gene: *N* gene of SARS-CoV-2; *RdRP*: gene for RNA-dependent RNA polymerase of SARS-CoV-2.

between the pool and individual results (Supplementary Figure 4) ($r(N) = 0.68$, $r(RdRP) = 0.7$, $r(E) = 067$; p value $<.001$). All primary samples from 136/759 positive pools (17.9%) were found to be negative when retested individually. One positive primary sample was found in 508 positive pools (66.9%), and ≥2 SARS-CoV-2 positive primary samples were found in 115 positive pools (15.2%) (Supplementary Figure 3).

Discussion

This retrospective cross-sectional evaluation shows that sample pooling is a feasible way of reducing resource and time expenditure during SARS-CoV-2 screening in a real-life setting. Using a two-stage pooling strategy, we tested 61,111 samples collected during two separate periods in different settings. While the collection of NPSs by clinicians followed by testing in single RT-PCR reactions remains the gold standard for SARS-CoV-2 detection, this process is costly and laborious. Our screening process for an asymptomatic population and large-scale episodic surveillance mitigates these problems by combining an alternative GL self-sampling procedure with pooled sample testing. Pooled sample testing for SARS-CoV-2 has previously been evaluated in several large-scale studies using both NPS samples

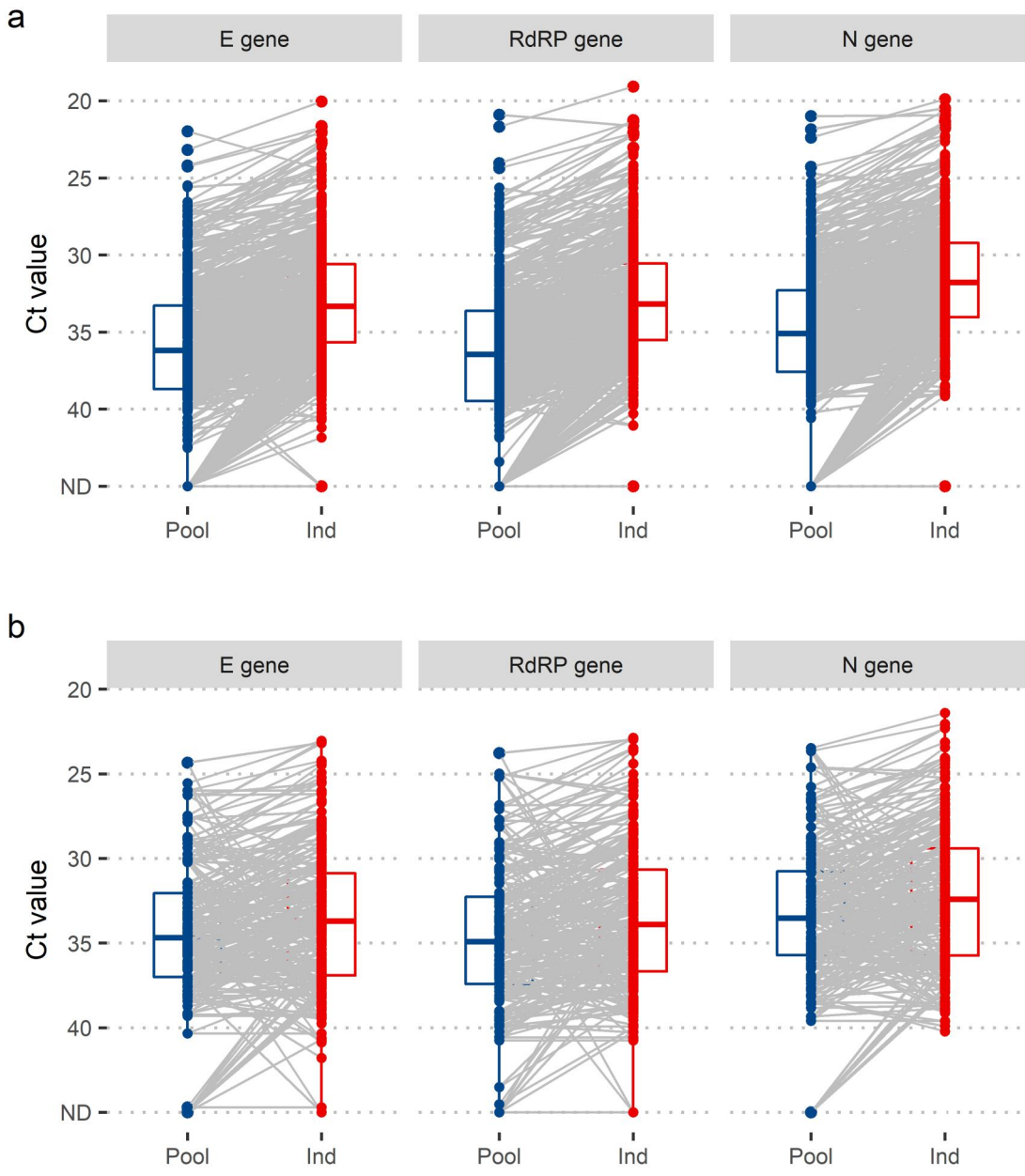

Figure 5. Paired RT-PCR results for individual (Ind) samples and six-sample pools (Pool) in arm B for pools with (a) only one SARS-CoV-2 positive sample and (b) ≥2 SARS-CoV-2 positive samples. Incomplete pools were excluded from the final analysis of paired results. *E* gene: *E* gene of SARS-CoV-2; *N* gene: *N* gene of SARS-CoV-2; *RdRP*: gene for RNA-dependent RNA polymerase of SARS-CoV-2.

collected by clinicians [18, 21,22] and self-collected saliva samples [23–25]. Moreover, a cross-sectional retrospective study using GL samples with five-sample pooling was conducted to assess the viability of self-sampling for large-scale surveillance (the study examined the results of >55,000 tests) of asymptomatic hospital healthcare workers (HCWs) with a SARS-CoV-2 prevalence below 1% [26]. Moreover, Thannesberger et al. [27] demonstrated the viability of self-sampling by GL and pool testing in statewide mass screening programs.

Diverse pooling strategies for detecting SARS-CoV-2 positive samples in pools of up to 32 samples have been described and evaluated, including pooling of viral media, pooling of collected swabs before RNA extraction, and pooling of RNA extracts [28–31]. Overall, it seems that pool size should be chosen based on the prevalence of SARS-CoV-2 in the population and its fluctuation in order to maintain the cost-effectiveness of sample pooling [32,33]. However, both our results and those of Barak et al. [21] indicate that pooled testing is an easily scalable resource-saving strategy if positivity rates oscillate between 0.5 and 6%. As we expected prevalence within this range and due to technical reasons, we introduced a pooling of 6 and 12 samples. While we used 12-sample pooling to screen the asymptomatic population, we switched to six-sample pooling

for the Covid-19 surveillance in schools to reach better efficacy since the national positivity rate significantly increased. Although we did not implement the pool size exactly according to the current positivity rate as recommended [32], we still reached cost-effectiveness and, more importantly, time-effectiveness better than expected. Once the infrastructure was set up, our algorithm demonstrated high flexibility and dynamic options in setting the pool size. However, we were not compelled to switch pool size dynamically because the positivity rate had not reached a threshold where the pooling approach ceased to be advantageous. The difference in positivity rate in both periods could have several reasons. First, in arm A, only asymptomatic patients were included for pooled testing, resulting in expected lower SARS-CoV-2 prevalence compared to overall national positivity. Regarding the second period, where pooled testing was implemented for Covid-19 surveillance at schools, it was already proposed that children are less susceptible to SARS-CoV-2 infection compared to adults [34,35]. Moreover, the national positivity rate includes all the positive tests in the Czech Republic, while our local positivity rate is ascertained only from samples tested in our centre, possibly resulting in those discrepancies.

In our study, sample pooling eliminated the need to perform 46,911 RNA isolation procedures and RT-PCR reactions, giving an efficiency consistent with previous reports [18, 21, 36]. Moreover, because pool testing is significantly less time-intensive than conventional testing using single reactions, its implementation in our laboratory more than doubled our testing capacity, to a maximum of 4200 samples per day. In fact, the laboratory's capacity exceeded the daily sampling capacity of the sampling point where the primary samples were collected. This made it possible to properly evaluate the potential and benefits of GL self-sampling, which has the important requirement of not requiring supervision by a HCW. Moreover, all GL samples could be collected simultaneously and delivered at once, avoiding potential limits on testing capacity. Recently published studies, including one from our group [14, 20, 37], have shown that the sensitivity and specificity of GL sampling are comparable to paired NPSs, making it an excellent option for Covid-19 screening and also for diagnostics of other respiratory infections.

While mass testing using sample pooling and self-sampling methods holds promise in containing potential future pandemics of a similar nature, it is imperative to assess the preparedness and requirements for establishing the necessary infrastructure. The sample processing outlined in this study involves a combination of automated procedures and optimised processing techniques, including the use of liquid handlers and simplified storage of primary samples. Although the utilisation of high-volume plates for primary sample storage has minimal impact on overall expenses, the procurement costs associated with liquid handlers may present a significant barrier to achieving the efficiency demonstrated in this study. Nonetheless, storing primary samples in labelled reserve plates alleviates the strain on storage capacity, ensuring precise sample organisation for high-throughput testing and facilitating their reuse for rapid retesting.

The main problem with automated processing and pooling by liquid handlers was the low fluidity of some samples. These samples were challenging to pipet and were a significant source of a possible contamination. False positivity was observed in both study cohorts but mainly in arm B, where false positives comprised 17.9% of the positive pools. The Ct values for the false positive pools were very high (Ct $>$38 for all three targeted SARS-CoV-2 genes). Only one SARS-CoV-2 gene was detected in 78.7% (107/136) of the arm B false positive pools, while two or all three SARS-CoV-2 genes were detected only in 11.0% (15/136), and 10.3% (14/136) of the false positive pools, respectively. We attributed the high false positivity rate in arm B to contamination resulting from the high daily testing capacity and elevated SARS-CoV-2 viral loads during the omicron Covid-19 outbreak. Another contributing factor was the use of relaxed criteria for identifying SARS-CoV-2 positive pools, which only required positivity for one gene and a Ct value $\leq$41. Applying more stringent criteria used in the evaluations of individual primary samples to the arm B pooled samples reduced the false positivity rate to just 6.2% (47/759). However, the original false positivity rate and the impact of evaluation criteria used for pool testing are similar to those reported elsewhere [38].

GL self-sampling combined with pooled RT-PCR testing was used to reduce the risk of SARS-CoV-2 transmission among students in several schools in place of rapid antigen testing, which was deployed nationally in schools at the time. Testing was performed weekly on Mondays or Tuesdays as students returned to school after the weekend. Receiving between 1200 and 3100 samples per day, the median delivery time of test results was less than 10 h from sample collection. Although samples were combined blindly, it was done in order as they were delivered in the laboratory and possibly might

increase the efficiency of pool testing. As published elsewhere, combining samples from the same school or social group seemed beneficial to increase the efficiency as testing samples from individuals with similar probabilities of SARS-CoV-2 infection can prevent unnecessary retesting [18, 21, 36]. In total, 762 students tested positive for SARS-CoV-2 during two months of testing in primary and secondary schools, preventing outbreaks and complete school closure. Testing at three-day or weekly (as described here) intervals was considered adequate to prevent new SARS-CoV-2 outbreaks if same-day delivery of results and isolation of SARS-CoV-2 positive individuals could be ensured [39]. These testing frequencies were also used in a prospective multicentre study that tested the feasibility of SARS-CoV-2 surveillance using saliva swabs obtained by the 'Lolli method' in primary and secondary schools [40]. Additionally, Vander Schaaf et al. [23], developed and implemented a testing procedure for Covid-19 surveillance at schools that had similar testing frequency and result delivery criteria; although their protocol called for saliva samples instead of GL, the reported turnaround time (8 h) for testing 930 students was comparable to that in our study.

In Covid-19 screening, test accessibility, testing turnaround time and frequency are more important considerations than test sensitivity [39]. Nevertheless, losses of sensitivity due to sample dilution are a central concern when considering the implementation of sample pooling. Therefore, a proper validation study should be conducted before switching to sample pooling. Mahmoud et al. [41] reported a pronounced loss of sensitivity when using four- and eight-sample pooling for Covid-19 mass screening, and found that the sensitivity of an assay targeting a single SARS-CoV-2 gene (*ORF1ab*) fell below 50% in weakly positive samples when using sample pooling. Our validation study similarly showed that not all RT-PCR assays are suitable for use with pooled samples; only those with sufficient sensitivity to detect viral genes in weakly positive pools (>35 Ct) should be considered. When using an assay satisfying this requirement, we observed only a minimal loss of sensitivity; the Ct value shift when comparing pooled and single samples was <1 Ct value shift among pooled and single reactions. However, when this assay was applied to real samples, it exhibited a loss of sensitivity consistent with previous reports [21–23, 29]: analysis of Ct value shifts for both validation and real data revealed a loss of sensitivity when comparing pooled and single samples but not when comparing 6-sample and 12-sample pools. This is consistent with the results of Chen et al. [29], who obtained similar results when comparing pools of 6 and 10 samples. These findings indicate that dilution may have only a minor effect on sensitivity when using sample pooling strategies.

Our study has several strengths. Most notably, it uses real-world data from an implementation of pooled testing for SARS-CoV-2 surveillance in outpatients using an alternative sampling strategy to increase testing capacity and lessen the burden on HCWs. The results obtained demonstrate the viability of the pooling strategy and show that it can be implemented relatively easily with minimal loss of sensitivity while providing a substantial increase in sample throughput. Moreover, its successful long-term use in this study shows that it can be incorporated sustainably into routine laboratory operations and is suitable for daily or episodic testing, such as in schools. Pooled testing of such large numbers of samples (>3000 samples per day) is also logistically challenging because the samples have to be preserved until deconvolution and SARS-CoV-2 positive sample tracing can be done. We therefore developed a simplified laboratory process that includes immediate sample inactivation to facilitate handling, automated robotic pipettors for liquid handling, and primary sample preservation in 96-deep well plates, enabling easy sample tracking and retesting while saving storage and working space. The major limitations of pool testing procedures are sample transport management and an increased possibility of missing true positive samples because incorrectly collected samples can be masked within pools. Despite these issues, our results suggest that pooling may be useful in a wide range of public health surveillance strategies and disease monitoring programs including SARS-CoV-2 testing.

In conclusion, we have clearly shown that pooled testing has significant advantages in Covid-19 surveillance. Pooled testing has resulted in considerable cost savings and increased testing capacity with minimal loss of sensitivity. Moreover, the implementation of GL collection has not only reduced demands on HCWs but also facilitated remote self-sampling at schools, optimising testing efficiency.

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Author contributions

All authors contributed to the study conception and design. Ondrej Bouska performed experiments and wrote the manuscript; Katerina Kubanova, Sona Gurska and Hana Jaworek performed experiments; Jana Vrbkova, Rastislav Slavkovsky and Petr Dzubak analysed and interpreted the data; Vladimira Koudelakova contributed to manuscript preparation, supervised the analysis, and critically revised the manuscript; Marian Hajduch secured funding and designed the experiment. Vladimira Koudelakova is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors agree with the article submission. All authors read and approved the final manuscript.

Disclosure statement

Marian Hajduch and Petr Dzubak declare that they are owners of IntellMed stock. Other authors have no relevant financial or non-financial interests to disclose.

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ORCID

Vladimira Koudelakova http://orcid.org/0000-0001-5842-7478
Marian Hajduch http://orcid.org/0000-0002-4834-908X

Data availability statement

Data available at https://privatecloud.imtm.cz/s/OzrrBb2ceZU9wgb

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