EXPERIMENTAL ARTICLES

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SALIVARY BILE ACIDS AS POTENTIAL NONINVASIVE MOLECULAR INDICATORS OF DUODENOGASTROESOPHAGEAL REFLUX EXPOSURE

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Achalasia of the cardia and hiatal hernia share overlapping symptoms and require clarification of their underlying pathophysiological mechanisms, including a possible duodenogastroesophageal component of reflux. Saliva is an accessible, noninvasive biological medium that may reflect refluxate components and compensatory clearance responses.

Aim. To compare mixed saliva parameters in a control group, in patients with achalasia, and in patients with hiatal hernia, and to identify the parameters most informative for assessing refluxate exposure and protective response.

Materials and Methods. A comparative study was conducted in three independent groups: controls, achalasia (ICD-10 K22.0), and hiatal hernia (ICD-10 K44). Unstimulated mixed saliva collected in the morning under fasting conditions was analyzed. Salivary portion volume, pH, pepsin, glycoproteins, total calcium, NOx, and bile acids were determined. Between-group differences were assessed using the Kruskal-Wallis test followed by pairwise comparisons with Dunn's test and Bonferroni correction, or using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post hoc test.

Results. Two parameters were the most informative. Saliva volume was higher in patients with achalasia and hiatal hernia than in controls, with no difference between the clinical groups, suggesting an enhanced salivary clearance response. The concentration of bile acids in saliva was increased in both clinical groups relative to controls. It did not differ between achalasia and hiatal hernia, consistent with a possible contribution of the duodenal reflux component and indicating exposure to refluxate components. Salivary pH, pepsin, glycoproteins, total calcium, and NOx showed no statistically significant between-group differences, highlighting the specificity of changes in salivary parameters.

Conclusions. Saliva volume and bile acids are promising noninvasive indicators that reflect the combined effects of refluxate exposure and compensatory protective mechanisms in esophageal diseases and may provide a basis for the further development of saliva-based biomarker strategies.

Keywords: biomarkers, noninvasive tests, bile acids, achalasia, hiatal hernia, duodenogastroesophageal reflux, saliva-based biomarkers.

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Achalasia and hiatal hernia (HH) are clinically significant esophageal disorders that often present with overlapping symptoms, including regurgitation, retrosternal discomfort, heartburn, and dysphagia. Despite overlapping symptom profiles, achalasia and HH represent distinct pathophysiological entities, and symptom-based attribution of underlying mechanisms is therefore inherently limited. In this setting, objective noninvasive biomarkers may serve as an adjunct to instrumental diagnostics by improving mechanistic interpretation when clinical signals are non-specific. Saliva represents a readily accessible biological matrix for such analyses, as it can be collected with minimal burden and may contain analytes reflecting both refluxate contact and elements of mucosal clearance.

For achalasia, high-resolution manometry remains central to diagnosis because it defines the underlying motor phenotype and informs therapeutic decision-making [1, 2]. Importantly, regurgitation in achalasia should not be equated with gastroesophageal reflux, as it may arise from esophageal retention and stasis. This confounder complicates symptom interpretation and supports analytical approaches that explicitly separate markers of refluxate exposure from markers related to impaired esophageal emptying.

Hiatal hernia is considered an important anatomical factor contributing to insufficiency of the anti-reflux barrier, and it is associated with an increased frequency and duration of reflux episodes as well as greater exposure of the esophageal mucosa to gastric contents. However, this condition appears pathophysiologically heterogeneous, because variation in hernia size, esophageal motor function, inflammatory activity, and tissue remodeling can plausibly translate into marked interindividual differences in both symptom burden and biomarker profiles. In practice, this heterogeneity complicates the interpretation of typical reflux complaints and makes any single marker less likely to perform uniformly across patients.

Current recommendations emphasize that symptom reports may not reliably track true reflux exposure, so objective verification generally requires instrumental assessment, including pH-impedance monitoring [3, 4]. At the same time, these investigations remain invasive and technically demanding, and their availability in routine settings is variable, consistent with the continuing need for complementary noninvasive approaches to estimate reflux exposure.

An important component of current concepts of reflux-associated injury is the composition of the refluxate. Beyond acid, components of duodenal contents, particularly bile acids, are likely to contribute to pathogenesis: they can plausibly amplify inflammatory and cytotoxic mucosal responses and thereby contribute to symptom persistence in mixed or non-acid reflux. This perspective is consistent with contemporary clinical evidence indicating that non-acid reflux components play a substantial role in symptom generation and mucosal injury, particularly in heterogeneous reflux phenotypes [5]. This is consistent with the limitation that acid exposure alone does not always capture the full reflux-related burden.

Against this background, there is growing interest in markers that reflect a “duodenal contribution” to the refluxate. Evidence for bile acids in saliva remains limited, yet available pilot data support the feasibility of quantitative measurement and suggest an association with clinical reflux phenotypes [6]. At the same time, systematic comparative studies in patients with achalasia and HH are virtually absent.

Saliva represents a convenient, noninvasive biological medium that can reflect both mucosal contact with refluxate components, including pepsin and bile acids, and compensatory protective responses. In this setting, the latter typically manifests as measurable shifts in salivary volume, buffering capacity, and clearance-related mechanisms. Hypersalivation is regarded as a reflex response to mucosal irritation and may serve a protective function by reducing the duration of epithelial exposure to harmful agents.

Pepsin remains the most extensively studied “reflux” salivary marker; nonetheless, systematic reviews and meta-analyses point to substantial variability in its diagnostic performance, with estimates shifting as a function of assay methodology and the criteria used to verify reflux. This variability has led to increasing recognition that single-marker approaches may be insufficient, and that combined salivary biomarker strategies could provide improved diagnostic accuracy in reflux-related conditions [7].

That interpretive caution extends to the preanalytical phase, where salivary results can be strongly influenced by collection conditions (fasting vs postprandial), time of day, concomitant medication therapy, and sample handling and storage [8, 9]. In practice,

this means that any comparison across groups or studies is only as credible as the underlying standardization of sampling and processing. This limitation is further supported by recent analytical evidence indicating that preanalytical variability remains a critical determinant of the performance and reproducibility of salivary biomarkers, particularly in reflux-related conditions [10].

Against this background, the evidence base for salivary bile acids is still comparatively sparse; however, available pilot data support the feasibility of quantitative measurement and suggest potential utility as indicators of refluxate exposure in patients with symptomatic reflux phenotypes [6].

Thus, despite the availability of instrumental methods for reflux assessment, there remains a need to identify simple noninvasive markers that could reflect both mucosal exposure to components of duodenal refluxate and compensatory protective responses of the salivary glands. Comparative data on bile acid levels and salivary volume in patients with achalasia and HH are limited, which underscores the relevance of the present study.

The study aimed to compare salivary bile acid levels and salivary volume in healthy controls, patients with achalasia, and patients with hiatal hernia to evaluate the potential of these measures as noninvasive biomarkers of duodenogastroesophageal reflux exposure and the associated compensatory salivary responses.

Materials and Methods

Study design and groups. The study was a comparative observational study and included three independent groups: a control group, patients with achalasia of the cardia (ICD-10 K22.0), and patients with hiatal hernia (HH; ICD-10 K44). The biomaterial was unstimulated mixed saliva collected in the morning under fasting conditions according to a standardized protocol, followed by centrifugation, aliquoting, and storage until analysis [11].

Diagnoses were verified based on clinical and instrumental examination in accordance with the local clinical protocol. The study was exploratory, designed to identify salivary parameters that are associated with distinct clinical phenotypes rather than to test a single prespecified causal hypothesis. Given the unequal group sizes and the evident variability in the data distribution, statistical inference was based on methods consistent with the data's distributional properties, with parametric or nonparametric approaches applied as appropriate.

Analyses were performed using an available-case approach without imputation of missing values; the effective sample sizes varied across variables and are reported in the corresponding tables.

The study was conducted in accordance with the principles of the Oviedo Convention (1997) and the Declaration of Helsinki (2013). The protocol was approved by the Biomedical Ethics Committee of the State Institution "Institute of Gastroenterology of the National Academy of Medical Sciences of Ukraine" (Protocol No. 2, 07 April 2022). Written informed consent was obtained from all participants.

Biochemical assays. Total calcium was measured using a colorimetric method with the "Filisit-Diagnostics" reagent kit. Stable nitric oxide metabolites (NOx) were quantified via nitrate-to-nitrite reduction followed by diazotization using the Griess reaction, as described by [12]. Glycoprotein concentration was assessed according to the protocol outlined in the methodological recommendations [11]. The study was conducted in accordance with the principles of the Oviedo Convention (1997) and the Declaration of Helsinki (2013). The protocol was approved by the Biomedical Ethics Committee of the State Institution "Institute of Gastroenterology of the National Academy of Medical Sciences of Ukraine" (Protocol No. 2, 07 April 2022). Written informed consent was obtained from all participants.

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Statistical analysis. Descriptive statistics were summarized according to data distribution: parametric variables are presented as mean ± standard deviation (M ± SD), whereas nonparametric variables are reported as median with the interquartile range, Me (Q1–Q3), where Q1 and Q3 represent the 25th and 75th percentiles, respectively. Normality was evaluated within each group

using the Shapiro–Wilk test. The choice of parametric versus nonparametric methods was based on normality testing and unequal group sizes [14].

For comparisons across three independent groups when variables were not normally distributed, the Kruskal–Wallis test was used. When a significant overall effect was observed, post hoc pairwise comparisons were conducted using Dunn's test with a Bonferroni adjustment for multiple testing ($\alpha^* = 0.0167$ for three pairwise contrasts).

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For variables meeting the assumptions of parametric analysis, one-way analysis of variance (ANOVA) was used, followed by Tukey's honestly significant difference (HSD) post hoc test to control the family-wise type I error rate in multiple pairwise comparisons [15].

All tests were two-sided, and differences were considered statistically significant at $P < 0.05$, with appropriate adjustment for multiple comparisons where applicable.

Generative large language model tools were used solely for language editing and manuscript structuring. All scientific content, analyses, and interpretations were produced and verified by the authors.

Results and Discussion

Comparative analysis indicated that, among the salivary parameters examined, salivary volume and bile acid concentration showed the most pronounced between-group differences (Table 1). Both measures were significantly higher in patients with achalasia and HH than in controls, which may reflect a combination of reflux exposure and compensatory responses of the salivary glands.

Comparative analysis of the additional salivary parameters did not reveal statistically significant between-group differences in pH, pepsin, glycoproteins, total calcium, or NOx levels (Table 2). At the same time, some measures, most notably pepsin and NOx, showed substantial inter-individual variability, which may reflect clinical phenotype heterogeneity and the limited specificity of these parameters as potential biomarkers.

Thus, the selectivity of the between-group effects indicates the relative specificity of salivary volume and bile acid levels as potential noninvasive indicators of duodenogastroesophageal reflux exposure and the associated compensatory salivary responses. The results obtained support the use of saliva as a biological matrix for identifying biomarkers of reflux-associated conditions.

The volume of a saliva portion differed statistically significantly among the three groups (Kruskal–Wallis test, $P = 1.28 \times 10^{-4}$, $\varepsilon'' = 0.115$), corresponding to a moderate effect size. Post-hoc analysis (Dunn test with

Table 1. **Key salivary indicators in the control, achalasia, and hiatal hernia (HH) groups and results**

Variable	Control, n	Achalasia, n	HH, n	Test (3 groups)	P value	Post hoc (adjusted P)
Salivary portion volume, mL	7.00 (5.25–9.50), $n = 14$	16.50 (10.00-20.00), $n = 20$	18.00 (10.00-22.00), $n = 108$	Kruskal-Wallis	1.28×10^{-4}	Control-Achalasia 5.83×10^{-4}; Control-HH 2.78×10^{-5}; Achalasia-HH 9.74×10^{-1} (Dunn, $\alpha^* = 0.0167$)
Bile acids, μmol/L	52.93 ± 28.02, $n = 14$	105.74 ± 42.80, $n = 20$	98.57 ± 30.04, $n = 107$	One-way ANOVA	3.03×10^{-6}	Control-Achalasia 1.52×10^{-5}; Control-HH 4.48×10^{-6}; Achalasia-HH 6.28×10^{-1} (Tukey HSD)

Note. Data are presented as Me (Q1–Q3) for nonparametric variables and as mean ± SD for parametric variables. Normality of the distribution was assessed using the Shapiro–Wilk test; the choice of statistical tests was based on the data's distributional properties and unequal group sizes. For overall between-group comparisons, the Kruskal–Wallis test or one-way analysis of variance (ANOVA) was used. If the overall effect was significant, post hoc pairwise comparisons were conducted using Dunn's test with Bonferroni adjustment ($\alpha^* = 0.0167$) or Tukey's HSD test. Statistical significance was set at $P < 0.05$, with correction for multiple comparisons. Missing data were not imputed.

Table 2. **Additional salivary parameters in the control, achalasia, and hiatal hernia (HH) groups and results of between-group comparisons (test Kruskal-Wallis)**

Variable	Control, n	Achalasia, n	HH, n	*P* value
Salivary pH	6.70 (6.54-7.00), $n = 18$	6.80 (5.80-7.18), $n = 20$	6.75 (6.35-7.36), $n = 108$	0.84
Pepsin, μg/mL	11.43 (9.23-14.23), $n = 16$	10.63 (7.19-67.53), $n = 20$	12.50 (8.75-17.00), $n = 109$	0.75
Glycoproteins, mg/mL	0.08 (0.06-0.13), $n = 14$	0.06 (0.04-0.12), $n = 19$	0.07 (0.04-0.11), $n = 108$	0.35
Total calcium, mmol/L	1.34 (1.18-2.74), $n = 22$	1.45 (1.18-1.68), $n = 20$	1.68 (1.33-2.33), $n = 108$	0.25
NOx, μmol/L	8.06 (6.03-9.92), $n = 14$	9.92 (4.41-31.99), $n = 18$	11.76 (4.41-39.71), $n = 106$	0.50

Note. Data are presented as Me (Q1–Q3). Between-group differences were assessed using the Kruskal-Wallis test. Differences were considered statistically significant at $P < 0.05$. Analyses were performed using an available-case approach; the number of observations (n) varied across variables due to missing values; no imputation was performed.

Bonferroni correction, $\alpha^* = 0.0167$) showed that in patients with both achalasia and HH, the value was higher than in the control group, whereas no differences were detected between achalasia and HH (Table 1). Based on median values, the volume of a salivary volume exceeded the control approximately 2.4-fold in achalasia and 2.6-fold in HH, indicating a potentially shared mechanism underlying enhanced salivary secretion in different esophageal diseases. Pathophysiologically, increased salivary volume may be interpreted as part of a compensatory protective response that enhances esophageal clearance and buffering of irritative exposures. This view is consistent with contemporary concepts of gastroesophageal reflux disease, which emphasize saliva as a key component of pre-epithelial defense and refluxate clearance; persistent exposure may, in turn, sustain reflex upregulation of salivary secretion [3,16]. In achalasia, a similar phenotype may also be associated with a combination of regurgitation, stasis, and mucosal irritation, which clinically manifests as a sensation of "excess saliva" and may be part of the disease's symptom complex [1, 2].

Salivary bile acid concentration showed a significant overall effect of group (one-way analysis of variance, $P = 3.03 \times 10^{-6}$; $\eta'' = 0.168$; $\omega^2 = 0.155$). Post hoc pairwise comparisons using Tukey's HSD test indicated significant differences between the control group and each clinical group, whereas no difference was observed between achalasia and HH. Based on mean values, the parameter in the achalasia and HH groups was higher relative to the control by approximately 86-100%, which suggests a contribution of the duodenogastroesophageal component of reflux, in which bile acids act as biologically active molecules that potentiate injury and inflammatory responses [3, 4].

The observed differences in salivary bile acids are consistent with reports supporting the feasibility of quantifying bile acids in saliva and their potential diagnostic relevance in symptomatic reflux phenotypes [6]. In HH, this finding may be related to impaired barrier function at the esophagogastric junction [17], whereas in achalasia, it may be attributable to conditions that favor retrograde movement of luminal contents and prolonged mucosal contact with injurious components secondary to impaired clearance [1]. At the same time, the same direction of changes in achalasia and HH, in the absence of differences between them, supports the view that bile acid measurement may serve as a marker of exposure to or contact with refluxate rather than a strictly nosological feature.

A similar pattern of changes in achalasia and HH may indicate shared disruption of barrier and clearance mechanisms at the esophagogastric junction, where coordinated function of the lower esophageal sphincter and the diaphragmatic sphincter component is physiologically important [18]. At the same time, evidence that HH is relatively uncommon in achalasia underscores that overlapping symptoms do not necessarily reflect a shared anatomical substrate, which, in turn, supports the value of independent markers of refluxate exposure [19].

Salivary acidity did not differ between groups, consistent with saliva's strong

buffering capacity; moreover, a single pH measurement may be insufficiently sensitive to episodic reflux exposure and is strongly influenced by sampling conditions [9]. Pepsin likewise did not show consistent between-group differences (Table 2) despite substantial variability. This corresponds to the literature, which indicates that the diagnostic performance of salivary pepsin is variable and depends on the protocol (timing and frequency of sampling, diagnostic criteria, symptom phenotype) [20]. Similarly, the lack of statistically significant between-group differences in NOx and selected "protective" components (glycoproteins and calcium), despite the presence of outliers, may reflect a combination of biological heterogeneity and susceptibility to pre-analytical conditions and relevant confounders. This pattern warrants cautious interpretation and suggests that future work would benefit from a more rigorous design and tighter standardization of sampling and analytical workflows [8, 9].

Overall, the obtained data indicate two key directions for noninvasive stratification, namely markers of refluxate composition (bile acids) and indicators of the compensatory clearance response (salivary portion volume). The combination of these parameters aligns with contemporary concepts of gastroesophageal reflux disease and reflux-associated conditions, in which symptom expression is shaped not only by acidity but also by refluxate composition and the efficiency of clearance mechanisms [3, 16]. In a broader context, the results obtained support the promise of a saliva-based biomarker approach as a basis for the further development of noninvasive analytical strategies in health biotechnology. This approach is consistent with recent studies suggesting that combinations of salivary biomarkers may offer improved diagnostic performance compared with individual markers alone, particularly in heterogeneous reflux phenotypes [7].

Conclusions

Salivary volume was significantly increased in patients with achalasia and hiatal hernia compared with controls, suggesting a role for the salivary component in esophageal clearance and protective responses. Salivary bile acids differed significantly between the control and clinical groups, suggesting a contribution of the duodenal component of reflux to symptom pathophysiology and indicating mucosal exposure to refluxate constituents. By contrast, salivary pH, pepsin, glycoproteins, total calcium, and NOx did not show consistent between-group differences in the present dataset, underscoring the selectivity of salivary changes and the potential specificity of the identified biomarkers. The pronounced variability of several measures likely reflects clinical heterogeneity across the studied conditions and highlights the need for rigorous standardization of saliva collection and analytical protocols. The most promising direction for further research appears to be the development of a noninvasive biomarker panel combining markers of refluxate exposure (bile acids) with parameters of compensatory salivary secretion (salivary volume), which may serve as a basis for advancing saliva-based analytical strategies in health biotechnology.

Ethical Standards

The study was conducted in accordance with the Oviedo Convention (1997) and the Declaration of Helsinki (2013) and was approved by the institutional bioethics committee (Protocol No. 2, 07 April 2022). Written informed consent was obtained from all participants.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

A.M. Halinska — experimental work, statistical analysis, and manuscript preparation; O.V. Severynovska — study conceptualization and manuscript editing. All authors approved the final version of the article.

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ЖОВЧНІ КИСЛОТИ СЛИНИ ЯК ПОТЕНЦІЙНІ НЕІНВАЗИВНІ МОЛЕКУЛЯРНІ ІНДИКАТОРИ ЕКСПОЗИЦІЇ ДУОДЕНОГАСТРОЕЗОФАГЕАЛЬНОГО РЕФЛЮКСУ

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Вступ. Ахалазія кардії та грижа стравохідного отвору діафрагми супроводжуються перекривними симптомами й потребують уточнення механізму ураження, зокрема можливого дуоденогастроезофагеального компонента рефлюксу. Слина є доступним неінвазивним середовищем, здатним відображати контакт із компонентами рефлюктату та компенсаторні реакції кліренсу.

Мета. Порівняти показники змішаної слини в контрольній групі, у пацієнтів з ахалазією та у пацієнтів із грижами стравохідного отвору діафрагми, виокремивши параметри, найбільш інформативні для оцінювання експозиції рефлюктату та захисної відповіді.

Матеріали й методи. Проведено порівняльне дослідження у трьох незалежних групах: контроль, ахалазія K22.0 та грижі стравохідного отвору діафрагми K44. Аналізували нестимульовану змішану слину, зібрану вранці натще. Визначали об'єм порції слини, pH, пепсин, глікопротеїни, загальний кальцій, NOx та жовчні кислоти. Міжгрупові відмінності оцінювали за допомогою критерію Краскела-Волліса з подальшими попарними порівняннями за тестом Данна та поправкою Бонферроні або однофакторного дисперсійного аналізу (ANOVA) з апостеріорним тестом Тьюкі (HSD).

Результати. Найбільш інформативними виявилися два показники. Об'єм порції слини був вищим у пацієнтів з ахалазією та ГСОД порівняно з контролем за відсутності відмінностей між клінічними групами, що відповідає посиленню кліренсної відповіді слиновиділення. Концентрація жовчних кислот у слині була підвищеною в обох клінічних групах відносно контролю й не відрізнялася між ахалазією та ГСОД, що узгоджується з можливим внеском дуоденального компонента рефлюксу та відображенням експозиції рефлюктату. pH слини, пепсин, глікопротеїни, загальний кальцій і NOx статистично значущих міжгрупових відмінностей не демонстрували, що підкреслює вибірковість змін слинних параметрів.

Висновки. Об'єм порції слини та жовчні кислоти є перспективними неінвазивними показниками, що відображають поєднання експозиції рефлюктату та компенсаторно-захисних механізмів при захворюваннях стравоходу й можуть розглядатися як основа для подальшого розвитку стратегій біомаркерної діагностики на основі слини.

Ключові слова: біомаркери, неінвазивні тести, жовчні кислоти, ахалазія, грижа стравохідного отвору діафрагми, дуоденогастроезофагеальний рефлюкс, слинні біомаркери.

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