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Editorial

LPS—Is It a Major Liability Factor for Cancer Risk and Severity?
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LPS—Is It a Major Liability Factor for Cancer Risk and Severity?

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Lipopolysaccharide (LPS) is a major integral component of the outer membrane of Gram-negative bacteria cell walls, that is released when bacteria lyse. LPS was shown to stimulate immune system cells by binding the cell-surface Toll-like receptor 4 (TLR-4) and activating transcription factors and protein kinases, such as NF-kB and p38 kinase. This in turn was shown to result in an increased production of proinflammatory cytokines and overexpression of cell adhesion molecules and matrix-degrading enzymes.1 The pathogenic role of LPS has been shown to be linked to several disease-states, viz., liver diseases, neurological degeneration, chronic inflammation of the gut, diabetes, and prostate cancer metastasis.2-4 One of our pioneering works showed increased circulatory levels of LPS and Zonulin as novel biomarkers of proinflammation in patients with type 2 diabetes. LPS is also proatherogenic factor and apart from its increase in blood levels in patients with atherosclerosis, LPS from Escherichia coli has also been shown to localize in carotid plaques, implying a mediatory role in atherosclerotic lesions.5

Is There an Association of Circulatory LPS and Risk of Advanced Colorectal Adenoma (ACA)?

In a recent study, Chen et al.7 investigated the association between LPS serum levels and risk of ACA. In that colonoscopy clinic-based study, male patients with diagnosis of ACA and polyp-free control individuals were phenotyped for comparative analyses with age, ethnicity, and information on demographics, lifestyle, and medical history along with serum levels of LPS measurement. Examining the LPS levels based on the characteristics and substratification of adenoma, the investigators showed that patients with large-sized adenoma (>2 cm), villous adenoma, or high-grade dysplasia tended to have higher serum levels of LPS compared to patients with smaller, tubular adenoma, and adenoma without high-grade dysplasia, respectively. Although LPS levels were slightly higher in patients with ACA compared to controls, the subtle differences in the LPS levels did not reach statistical significance. With the results from multiple regression analysis, the authors concluded that there was no statistically significant association between LPS and the risk of ACA. As both the groups in this study were equally confounded by obesity, diabetes and hypertension (conditions known to exhibit elevated levels of LPS), this finding could have been attributed to the lack of statistical significance in the LPS levels. However, authors have admitted that larger future studies are warranted to further investigate the association between the systemic levels of LPS and risk of colorectal tumors, and to gain a better understanding of the relevant clinical implications.

The Biological Link Between Gut Microbiota and Cancer Risk Attests to a Role for LPS

Recent studies imply that gut dysbiosis and leakage of bacterial components (majorly LPS), may contribute to the metabolic disturbances and systemic inflammation linked to several disease-states. More importantly, the gut microbiota has emerged as a central player that mechanistically links various risk factors to colorectal cancer (CRC) pathogenesis.8,9 While increased intestinal permeability is strongly linked to elevated level of gut microbiota-associated LPS-induced local inflammation, both of these phenomena were shown independently associated with CRC.10,11 In a multicohort CRC metagenome study, it was shown that the composition of specific bacteria enriched in CRC patients were also correlated to LPS-related pathway.12 These studies attest to a definite role of LPS as a mediator in the genesis of CRC and its progression to severity.

The Need for a Panel of Biomarkers to Assess CRC Risk

The Limulus amoebocyte lysate (LAL) test, an assay that is routinely used to quantify LPS as an endotoxin, has limitations due to the levels of LPS in the systemic circulation of healthy humans and within those with various clinical disorders, so that they can vary over a wide range.13 Because of its short half-life, low concentration, and high susceptibility to interfering substances, such as endogenous inhibitors of LPS, the utility of LPS detection via the LAL method has limitation in the routine clinical setting and large-scale studies.14 This definitely warrants the need for development of more reliable methods for LPS detection. In order to communicate with the innate immune system, LPS binds to the LPS-binding protein (LBP), which is pivotal for the binding of CD14 and transfer to the TLR4 complex.15 Therefore, LBP measurement is considered as a surrogate marker of metabolic endotoxemia. Indeed, an increased LBP level has been shown to be closely associated with obesity, prediabetes, and atherosclerosis,16-18 and has been observed as a significant and independent predictor of coronary artery disease.19 Moreover, LBP and CD14 polymorphisms

Abbreviations: ACA, advanced colorectal adenoma; CRC, colorectal cancer; IAP, intestinal alkaline phosphatase; LAL, Limulus amoebocyte lysate; LBP, LPS-binding protein; LPS, Lipopolysaccharide; TLR-4, Toll-like receptor 4.

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have been shown to be correlated with increased CRC risk in Han Chinese. While intestinal alkaline phosphatase (IAP) has now emerged as a new factor that is essential for maintaining proper gut homeostasis, the role of IAP in detoxifying LPS has been demonstrated in several studies.\textsuperscript{21,22}

Considering the above, it is important that studies that profile the biomarker role of circulatory levels of LPS in relation to disease-states should consider the LPS pathway approach and collectively measure a panel of circulatory biomarkers, viz., LPS, LBP, soluble CD14, Zonulin, and IAP. These potential biomarkers have to be correlated to conventional risk factors of CRC, so as to make further improvement(s) in disease prediction, treatment, and management. In this context, a recent study by Yin et al.\textsuperscript{23} used multiomics-based prognostic analysis and target-regulation simulation modeling and detected several prognosis risk biomarkers for colon adenocarcinoma (which includes LBP). Therefore, it is advised that an LPS pathway approach (rather than just measuring circulatory LPS levels) would be the ideal way not only to predict the disease-risk but also to unravel new perspectives for novel drug development and therapeutic applications for colorectal adenoma as well as other non-communicable diseases.

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Conflict of interest

The author has no conflict of interests related to this publication.

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Original Article

Introduction

Coronavirus disease 2019 (COVID-19) has been pandemic in the world.1–4 It has now affected more than 560,000 Americans.3,5 Several attempts were successfully made to model COVID-19 daily incidence in China.1,6 However, the trends of daily incidence and deaths of COVID-19 in the USA are still poorly understood.3,5

Trends and Prediction in Daily New Cases and Deaths of COVID-19 in the United States: An Internet Search-Interest Based Model

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Background and objectives: The daily incidence and deaths of coronavirus disease 2019 (COVID-19) in the USA are poorly understood. Internet search interest was found to be correlated with COVID-19 daily incidence in China, but has not yet been applied to the USA. Therefore, we examined the association of internet search-interest with COVID-19 daily incidence and deaths in the USA.

Methods: We extracted COVID-19 daily new cases and deaths in the USA from two population-based datasets, namely 1-point-3-acres.com and the Johns Hopkins COVID-19 data repository. The internet search-interest of COVID-19-related terms was obtained using Google Trends. The Pearson correlation test and general linear model were used to examine correlations and predict trends, respectively.

Results: There were 636,282 new cases and 28,325 deaths of COVID-19 in the USA from March 1 to April 15, 2020, with a crude mortality of 4.45%. The daily new cases peaked at 35,098 cases on April 10, 2020 and the daily deaths peaked at 2,494 on April 15, 2020. The search interest of COVID, “COVID pneumonia” and “COVID heart” were correlated with COVID-19 daily incidence, with 12 or 14 days of delay (Pearson’s $r = 0.978$, 0.978 and 0.979, respectively) and deaths with 19 days of delay (Pearson’s $r = 0.963$, 0.958 and 0.970, respectively). The 7-day follow-up with prospectively collected data showed no significant correlations of the observed data with the predicted daily new cases or daily deaths, using search interest of COVID, COVID heart, and COVID pneumonia.

Conclusions: Search terms related to COVID-19 are highly correlated with the COVID-19 daily new cases and deaths in the USA.

Keywords: Trend; Incidence; COVID-19; USA; Pandemic; Model; Search interest.

Abbreviations: CDC, Centers for Disease Control and Prevention; COVID-19, coronavirus disease 2019; WHO, World Health Organization.

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search-interest has been used for modelling and detecting influenza epidemics in the USA and Australia.11,12 We, therefore, aimed to examine the association of search-interest with daily incidence/new cases and deaths of COVID-19 in the USA, using population-based data and a semiparametric model.

Methods

The data of daily new cases and new deaths of COVID-19 in the USA were extracted from the 1-point-3-acres.com data repository on April 9, 2020, respectively, for modelling. We later obtained additional data from these sites to evaluate our models’ accuracies using Pearson’s correlation coefficients. We used a semiparametric model, including prediction of the daily new-case or new-death value based on a given Google Trends search-interest using Pearson’s correlation (the parametric component), as well as assigning such a predicted value to the corresponding date of the given Google Trends search interest. Owing to no finite dimensionality of Google Trends search-interest versus time, the second component thus is non-parametric.

Data from the World Health Organization (WHO) Situation Reports appeared significantly inconsistent, and thus were not used.13 According to the 1-point-3-acres.com website, their data were extracted from various media and government websites, have been manually verified,9 and have been used by various parties, including Johns Hopkins COVID-19 data repository, WHO, and many others. Due to the use of publicly available, de-identified data and lack of protected health information, the study is exempted from requiring an Institutional Review Board approval (Category 4).

We used the Google Trends function to extract the data of search-interest with the search period of March 1 to April 7, 2020 and COVID-19-related search terms. Based on the COVID-19 symptoms, common terms for COVID-19 and common diseases in the USA, we chose the search terms of “COVID-19,” “COVID,” “coronavirus,” “SARS-CoV2,” “pneumonia,” “high temperature,” “cough,” “COVID heart,” “COVID pneumonia,” and “COVID diabetes.” Google Trends search-interest represented search interest relative to the highest search-interest for a given time and region.7,12 A value of 100 is the peak popularity for the term, while a score of 0 means there were not enough data for this term.

We then examined the lag correlations of the terms’ search interests with COVID-19 daily new cases and deaths as described before,7 whereas the lag time was defined as the difference between a data point’s original corresponding time and the shifted one in the lag correlation study. The lag times of our interest were up to 20 days for daily new cases and 23 days for daily death, respectively. The terms with the top-3 correlation coefficients were used to build respective generalized linear models. Based on these models, we used the existing search interests to predict future COVID-19 daily new cases and new deaths in the USA, which would be compared with the prospectively collected data for assessing prediction accuracies.

All statistical analyses were carried out using Stata (version 15). The models’ accuracies were assessed using Pearson’s $r$. All $p$ values were two-sided. Only a $p < 0.05$ was considered statistically significant.

Results

The Johns Hopkins data repository and 1-point-3-acres.com provided slightly different estimates of COVID-19 daily new cases and deaths in the USA, although they claimed to share data. The data of a given date from 1-point-3-acres.com dataset varied by the release dates. Considering the data inconsistency, we chose the John Hopkins’ data for modelling, and the 1-point-3-acres.com data for a sensitivity study. There were 636,282 new cases and 28,325 deaths of COVID-19 reported in the USA from March 1 to April 15, 2020, with a crude mortality of 4.45%. The daily new cases peaked at 35,098 cases on April 10, 2020 and the daily deaths peaked at 2,494 on April 15, 2020.

Google Trends search-interests had a 2-day delay in reporting (i.e. a search on April 9 yielded data up to April 7). COVID-19 has a much lower search interest score than COVID (Fig. 1), and was excluded from additional analysis also owing to its close relationship with COVID. As reported before, the correlation coefficients of search terms changed with lag time (Fig. 2). Among the nine terms we searched, COVID, “COVID pneumonia” and “COVID heart” had the top-3 correlation coefficients for the correlation with daily incidence and new deaths (Table 1). Our predicted COVID-19 daily new cases and new deaths would plateau for about 12 days (Fig. 3), suggesting a possible 12-day plateau of these epidemiologic parameters in the future.

The sensitivity study using 1-point-3-acres’ data revealed the correlation coefficients that were similar to those produced using Johns Hopkins’ data (Table 1). The 7-day follow-up with prospectively collected data showed no significant correlations of the observed data with the predicted daily new cases using search-interest of COVID, COVID heart and COVID pneumonia ($p = 0.178, 0.480$ and $0.094$, respectively) nor with the predicted daily new deaths using search interest of COVID, COVID heart and COVID pneumonia ($p = 0.267, 0.222$ and $0.841$, respectively).

Discussion

This population-based study shows that there were 636,282 new cases and 28,325 deaths of COVID-19 reported in the USA from March 1 to April 15, 2020. It also shows that the search-interest of COVID, COVID pneumonia, and COVID heart were highly correlated with COVID-19 daily new cases and new deaths, with a delay of 12 days and 19 days, respectively. However, the prediction accuracies of these models appeared low during a 7-day follow-up.

To our knowledge, this study provided, for the first time, evidence that search-interest pertinent to COVID-19 is highly correlated with the trends in COVID-19 daily new cases and new deaths in the USA. The approximately 7 days of difference in lag time between daily new cases and deaths suggest the possibility of a 7-day interval between COVID-19 diagnosis and death in some patients. Additional studies are warranted to investigate this hypothesis. The findings of our study enable us to model daily new cases and deaths in the USA during the early phase (March 1 to April 8) of the COVID-19 outbreak and may greatly help prevent and prepare for any upcoming pandemic and burdens of COVID-19 in the future.

The 12 days of lag time in the USA, as shown by us, was longer than the previously reported 9 days in China.7 Several factors may contribute to this difference but should be subject to additional studies. First, there was a significant delay in testing for COVID-19 in the USA,14 which might subsequently lead to longer lag time between the trends of search-interest and daily incidence. Second, the U.S. Centers for Disease Control and Prevention (CDC) recommended a priority-based testing strategy and allowed for not testing some subjects considered low-priority when the COVID-19 tests are short in supply.15 The criteria for testing COVID-19 in the USA, therefore, were different from those in China and Europe.
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where the WHO criteria were adopted. Thus, the patients, who met the WHO criteria, may not be tested and subsequently not included in the daily incidence in the USA; this could lead to underreporting of daily incidence. Third, the biological and socio-economic differences between the USA and Chinese patients may also contribute to the difference. Finally, the prevalent COVID-19 subtypes in the USA may also be different from those in China and result in different lag times.

This study provides several lines of valuable evidence. First, COVID-19 daily new deaths in the USA are poorly understood, and are here described and studied using a semiparametric model. Second, we extensively examined nine COVID-19-related search terms, which are more than the two used in a previous study. Our data also suggest that pneumonia and heart problems were highly relevant to the daily new cases and deaths in the USA. This finding may be explained by the frequent pneumonia and cardiac injuries seen in COVID-19 patients. Third, the lag time in our study was longer than that previously reported in China (12 days vs. 9 days). However, the 12 and 19 days of lag time also afforded us the opportunity to assess a model’s prediction accuracy for a longer period of future trends. Fourth, the comparison of predicted values and prospectively collected data will significantly reduce the recall and selection biases.

We will continue updating the models’ accuracies as more data become available (see https://github.com/thezhanglab/COVID-US-google). Indeed, we found very high correlation in retrospective modelling but low accuracy in prediction, suggesting that the search-interest based model may be more helpful in predicting daily-incidence peak or early outbreak than post-peak or post-intervention trends. The unexpected low accuracy of model prediction was due to significant attenuation of trend plateau. It may be linked to the April 3 recommendation of wearing masks by the U.S. CDC, which was 5 days before our model’s peak time and matched the COVID19’s median incubation time of 5 days. Finally, to our knowledge, we are first to examine the correlations of search interest with the COVID-19 daily new cases and deaths in the USA and show greater correlations (Pearson’s $r > 0.97$) than reported in the Chinese data.

This study is limited by the retrospective nature of the modeling part and may have some related biases. Moreover, due to the different testing strategies and criteria used in the USA and other countries, the comparison of our findings to those of other countries should be interpreted with caution. Finally, the data from Johns Hopkins’ data repository was not independently validated or authenticated. However, our sensitivity study using the 1-point-3-acres’ data confirms a similar correlation of search-interest with COVID-19 daily new cases and deaths in the USA.

Future directions

Despite the high correlation coefficients in retrospective study/
Fig. 2. Lag correlations between Google Trends search-interest of the terms “COVID,” “COVID heart,” “COVID pneumonia,” and others, and the daily new cases and deaths of COVID-19 in the USA, March 1 to April 8, 2020. (a, c) The search terms with the highest Pearson’s correlation coefficients for daily new cases and new deaths, respectively; (b, d) The rest of the search terms.

modeling, the prediction-models based on the search-interest trend reached low accuracies during a 7-day follow-up. Additional studies are warranted to understand and improve these models. Why the prediction model failed should also be examined. The April 3 CDC recommendation of more indications for mask-use might be one of the reasons. Finally, the factors linked to and the epidemiological sig-

Table 1. The search term of the top-3 correlation coefficients for correlations with COVID-19 daily new cases and deaths, March 1 to April 8, 2020

<table>
<thead>
<tr>
<th>Search term</th>
<th>Johns Hopkins Data Repository</th>
<th>1-point-3-acres.com</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daily new cases</td>
<td>Daily new deaths</td>
</tr>
<tr>
<td></td>
<td>Days earlier</td>
<td>(r^2)</td>
</tr>
<tr>
<td>COVID heart</td>
<td>12</td>
<td>0.979</td>
</tr>
<tr>
<td>COVID pneumonia</td>
<td>14</td>
<td>0.978</td>
</tr>
<tr>
<td>COVID</td>
<td>12</td>
<td>0.978</td>
</tr>
<tr>
<td>Cough</td>
<td>19</td>
<td>0.932</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>19</td>
<td>0.914</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>19</td>
<td>0.848</td>
</tr>
<tr>
<td>COVID diabetes</td>
<td>18</td>
<td>0.821</td>
</tr>
<tr>
<td>SARS-CoV2</td>
<td>18</td>
<td>0.814</td>
</tr>
<tr>
<td>High temperature</td>
<td>17</td>
<td>0.681</td>
</tr>
</tbody>
</table>

*aThe highest correlation coefficients among the correlation coefficients of a given search term by various lag times.*
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**Conclusions**

This population-based observational study shows that search terms related to COVID-19 are highly correlated with the trends in daily new cases and new deaths of COVID-19 in the USA. Therefore, an internet search-interest based model may be used to predict development and peak-time of COVID-19 outbreak.

**Acknowledgments**

The data will be regularly updated at https://github.com/thezhanglab/.

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**Fig. 3. Google Trends search-interest and the trends in COVID-19 daily new cases and new deaths in the USA, March 1 to April 15, 2020.** (a–c) The search-interests of “COVID,” “COVID heart,” and “COVID pneumonia” in Google Trends were 12 to 13 days lagged from COVID-19 daily new cases/incidence (Pearson’s $r = 0.977$, 0.982 and 0.973, respectively, $p < 0.001$ for all). (d–f) The search interests of “COVID,” “COVID heart,” and “COVID pneumonia” in Google Trends were 19 to 20 days lagged from COVID-19 daily new deaths (Pearson’s $r = 0.967$, 0.977 and 0.972, respectively, $p < 0.001$ for all). Note, d12, d14 and d19 indicate the trend curves were shifted for 12, 14 and 19 days, respectively, to compensate for lag time. The 7-day follow-up with prospectively collected data showed no significant correlations of observed data with the predicted daily new cases using search interest of “COVID,” “COVID heart,” and “COVID pneumonia” search ($p = 0.178$, 0.480 and 0.094, respectively), or with predicted daily new deaths ($p = 0.267$, 0.222 and 0.841, respectively).
COVID-US-google, as new prospectively collected incidence data become available.

Funding

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

LZ 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. XY and JX contributed equally and should be considered co-first authors. Concept and design (LZ, NG); drafting of the manuscript (XY, JX); statistical analysis (XY, LZ); supervision (LZ); acquisition, analysis, or interpretation of data (all authors); critical revision of the manuscript for important intellectual content (all authors).

References


Serum Levels of Lipopolysaccharides and Risk of Advanced Colorectal Adenoma

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Abstract

Background and objectives: Production of lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria promotes the survival of cancer cells. Systemic level of LPS is considered a biomarker for microbial translocation. The association between LPS and the risk of colorectal tumors is not well known. The goal of this study was to examine the association between LPS serum levels and risk of advanced colorectal adenoma (ACA).

Methods: In this colonoscopy clinic-based case-control study, cases were male patients with a diagnosis of ACA, and controls were polyp-free male participants. Cases and controls were individually matched by age, ethnicity, and blood collection time. Information on demographics, lifestyle, and medical history was obtained using structured questionnaires. Serum levels of LPS were quantitated using the kinetic limulus amebocyte lysate assay. Multivariable conditional logistic regression model was used to estimate the odds ratio and its 95% confidence interval of the ACA in association with serum LPS adjusting for cigarette smoking, body mass index, and medical history.

Results: We examined 43 cases and 43 paired controls, with a mean age of 62 years. There was no significant difference in serum LPS levels between the cases and controls (0.28 vs. 0.25 endotoxin units (EU)/mL, P = 0.58 for the non-parametric test). The adjusted odds ratio and its 95% confidence interval of ACA was 1.83 (0.40–8.24) in multivariable logistic regression model.

Conclusions: Serum levels of LPS were not statistically significantly associated with an increased risk of ACA in this preliminary study.

Keywords: Gut microbiome; Colorectal cancer; LPS; Endotoxin, Leaky gut; Microbial translocation; Biomarker.

Abbreviations: ACA, advanced colorectal adenoma; BMI, body mass index; CI, confidence interval; CRC, colorectal cancer; CV, coefficient of variation; EU, endotoxin units; LAL, limulus amebocyte lysate; LPS, lipopolysaccharides; MEDVAMC, Michael E. DeBakey VA Medical Center; MET, metabolic equivalent of task; OR, odds ratio; TLR, toll-like receptor.

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Introduction

The gastrointestinal tract is the habitat of numerous bacteria of which some are known pathogens, including B. fragilis, Clostridium, and certain strains of Escherichia coli. The microbiome has multiple functions in metabolism, mucosal integrity, and immunity. Prior studies have shown that an imbalance of the gut microbiome and the microbial metabolites can trigger dysregulation of various metabolic pathways that lead to colon cancer. For example, the Gram-negative anaerobic bacteria, Fusobacterium, are more abundant in colorectal cancer (CRC) tissue. Fusobacterium nucleatum creates an inflammatory environment that promotes the formation of colorectal tumors. Similarly, Escherichia coli cause genotoxic inflammation by attaching to the gut epithelium.
eral mediators for these oncogenic effects of the bacteria have been proposed. One of which is through lipopolysaccharides (LPS), which directly induce deleterious alterations of enterocyte membrane structure and function.

LPS, also referred to as endotoxins, are found in the outer membrane of Gram-negative bacteria. When Gram-negative bacteria undergo cell death or cell division, the bacterial membranes release LPS. Microbial translocation is defined as the passage of viable or non-viable microbes and microbial product such as LPS across an anatomically intact intestinal barrier to lamina propria and mesenteric lymph node, and possibly other tissue. The continuous presence of these products in the systemic circulation may lead to low-grade inflammation. As inflammation ensues, macromolecular LPS can be absorbed and frequently induce a toxic reaction by various receptor-mediated signals. Elevated LPS levels have been associated with induction of tumor necrosis factor alpha, leading to chronic inflammation and a weakened intestinal epithelial barrier, and appear to be pathogenic factors in disorders such as irritable bowel disease and necrotizing enterocolitis. Animal studies have also shown that LPS may accelerate inflammation-related aging. Therefore, systemic levels of LPS may be a biomarker of microbial translocation and colorectal adenomas or CRC.

While several studies have examined the association between serum levels of LPS or markers of microbial translocation and risk of developing CRC, few have studied the association between LPS and its precursor lesion, namely advanced colorectal adenoma (ACA). Adenomas are the products of proliferation from dysplastic nonmalignant epithelial cells, which can progress into adenocarcinoma as deleterious mutations accumulate. Therefore, we examined the association between serum levels of LPS and risk of developing ACA in a colonoscopy clinic-based case-control study using a kinetic assay. We hypothesized that serum levels of LPS are associated with an increased risk of ACA.

**Methods**

**Study design and study population**

The study cohort and comprehensive eligibility and exclusion criteria for this cross-sectional study have been previously reported. The study was approved by the Institutional Review Boards of Baylor College of Medicine and Michael E. DeBakey VA Medical Center (MEDVAMC). All participants provided written informed consent for the study. From 2013 to 2017, a total of 612 participants, aged 50 and 79 years, were recruited from the colonoscopy clinic at MEDVAMC. A total of 55 patients had a diagnosis of ACA, which was defined as a polyp >1 cm found on colonoscopy or a colorectal adenoma with a villous component or high-grade dysplasia pathologically. Participants in the control group had no colorectal polyps found during the colonoscopy and had no history of colorectal adenoma in the past 3 years. Each control was individually matched to each case according to age (±5 years), race/ethnicity, and sample collection time (±3 months; and morning or afternoon collection).

**Data collection**

Patients were enrolled either on the day they were educated for bowel preparation or on the day of their colonoscopy. Information on demographics, lifestyle, and medical history was obtained through an interviewer-administered lifestyle questionnaire. The detailed procedure has been previously reported. The pathologist (Dr. Daniel Rosen) obtained the pathological features of the adenoma/polyp. The number of polyps/adenoma, location and size of the lesion, hyperplastic polyps, and high-grade dysplasia were documented for each patient.

**Blood collection and LPS measurement**

Fasting blood samples were collected on the day of the colonoscopy. Serum used for the LPS assay was placed into 7 mL anticoagulant-free BD vacutainer glass tubes previously baked to eliminate possible LPS contamination, and aliquoted into an endotoxin-free glass vial that was baked. These samples were stored at −80 °C before testing for LPS using the quantitative Limulus Amebocyte Lysate (LAL) assay. The PYROGENT-5000 kinetic turbidimetric LAL assay (Lonza, Basel, Switzerland) is designed to test for endotoxin of Gram-negative bacteria in serum. The serum samples were diluted to a 1:10 ratio, heated, mixed with the LAL, and incubated for 10 m at 37 °C, and a stop reagent was added to stop the reaction. A substrate solution was mixed with LAL solution and incubated at the same temperature for 6 m. A yellow color appeared if the endotoxin was present in the serum. The absorbance of the sample was measured through a spectrophotometer at wavelength of 340 nm. These absorbance values were then converted to concentrations in EU/mL and reported. The sensitivity of the assay ranges from 0.01 to 100 EU/mL. One EU is defined as the biological activity induced in the endotoxin test by about 120 pg LPS from *E. coli* O113H10:K21.

**Quality control**

The matched case-control pairs were assayed in the same 96-well plate. Each sample was assayed in duplicate. We included 5% of duplicate samples in each batch to monitor the batch variation. After examining the duplicate data, we found 11 case-control pairs (of 55 cases-control pairs) had a high (>15%) coefficient of variation (CV) in either the case or control. These pairs were not included in the final analysis. We also eliminated one outlier of LPS value in controls. Therefore, we included 43 case-control pairs in the final analysis.

**Statistical analysis**

Data were analyzed using the STATA (StataCorp, College Station, TX, USA) software. The chi-squared test was used to test the difference in the distribution of categorical data between cases and controls. The paired t-test was used to test the difference in the means of continuous characteristics (e.g., body mass index [BMI] and physical activity) between cases and controls. We used the Wilcoxon signed-rank test to compare the means of LPS levels between cases and controls, because the LPS levels did not follow the normal distribution as tested by the Kolmogorov-Smirnov test. The natural log-transformed LPS values were generated for the analyses where the normal distribution assumption should be met. We examined the associated between covariates and normalized LPS levels using the linear regression. Univariate and multivariable conditional logistic regression were used to estimate the odds ratio (OR) and 95% confidence interval (CI) for ACA in association with the transformed LPS value as the continuous variable. The covariates in the multivariable regression model included smoking, alcohol use, BMI, hypertension, and type 2 diabetes. We
also modeled the LPS levels as a dichotomized variable using the median in the controls as the cutoff point. Among those who had adenoma, we further tested whether transformed LPS levels differ by the characteristics of ACA according to the number of adenoma (more than 2 ACA or not), size of the adenoma (<2 cm vs. ≥2 cm), villous component (have vs. not have), and dysplasia (high grade dysplasia present or not). A $P$-value < 0.05 indicated statistical significance in a two-sided test.

**Results**

All participants were men with a mean age of 62 years. There was no significant difference in the distribution of BMI, history of polyps, physical activity, history of diabetes and hypertension, or alcohol use between 43 cases and 43 controls (Table 1). There was an insignificantly higher proportion of current smokers in the cases than controls ($P = 0.09$). Among those who were found to have adenoma, 67% had one to two lesions, 19% had three to four lesions, and 14% had more than four lesions. A total of 29% had the size of adenoma greater than 2 cm, 37% adenoma had villous components, and 23% had high-grade dysplasia.

LPS was detected in all our samples. The absolute levels of LPS were slightly higher in cases (0.28 EU/mL) than controls (0.25 EU/mL) ($P = 0.23$) (Table 1). Figure 1 shows the pairwise comparison between the individually matched cases and controls using the untransformed value. Furthermore, the serum levels of LPS did not differ by smoking, alcohol use, and BMI in controls ($P > 0.05$) (data not shown). There was no statistically significant association between LPS and the risk of ACA (multivariable OR = 1.83, 95% CI: 0.40 to 8.24) (Table 2). We did not observe a significant association when LPS was modeled as a dichotomized variable (data not shown).

We further examined the LPS levels based on the characteristics of adenoma among patients with ACA (Fig. 2). Patients with a large sized adenoma (>2 cm), villous adenoma, or high-grade dysplasia

---

**Table 1. Characteristics of controls without polyps and cases with advanced colorectal adenomas**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls ($n = 43$)</th>
<th>Cases ($n = 43$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>62.9 (6.7)</td>
<td>61.9 (6.4)</td>
<td>0.43</td>
</tr>
<tr>
<td><strong>Men, %</strong></td>
<td>100</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Non-Hispanic white, %</strong></td>
<td>62.0</td>
<td>60.4</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>BMI, kg/m$^2$</strong></td>
<td>29.9 (5.5)</td>
<td>31.6 (8.8)</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>BMI in categories, kg/m$^2$, %</strong></td>
<td></td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td>&lt;25</td>
<td>25.58</td>
<td>18.60</td>
<td></td>
</tr>
<tr>
<td>25-&lt;30</td>
<td>25.58</td>
<td>27.91</td>
<td></td>
</tr>
<tr>
<td>≥30</td>
<td>48.84</td>
<td>53.49</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking status, %</strong></td>
<td></td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Never</td>
<td>44.2</td>
<td>23.2</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>37.2</td>
<td>44.2</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>18.6</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td><strong>Current alcohol drinking, %</strong></td>
<td>53.5</td>
<td>60.5</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>History of polyp, %</strong></td>
<td>18.6</td>
<td>20.9</td>
<td>0.79</td>
</tr>
<tr>
<td><strong>Physical activity, MET-minutes per week</strong></td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td>Median (interquartile range)</td>
<td>876 (198–3,066)</td>
<td>738 (169–3,066)</td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes, %</td>
<td>37.2</td>
<td>25.6</td>
<td>0.24</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>55.8</td>
<td>62.8</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>Lipopolysaccharide (EU/mL)</strong></td>
<td></td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>Median (interquartile range)</td>
<td>0.24 (0.19–0.31)</td>
<td>0.24 (0.18–0.35)</td>
<td></td>
</tr>
<tr>
<td>Mean (standard deviation)</td>
<td>0.26 (0.01)</td>
<td>0.28 (0.02)</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>Pathology of adenoma (median (range) or %)</strong></td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>No. of adenoma</td>
<td>2 (1–6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size of largest adenoma if multiple (cm)</td>
<td>1.2 (0.5–3.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Having high-grade dysplasia, %</td>
<td>23.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Having villous component, %</td>
<td>37.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serrated adenoma, %</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; EU, endotoxin unit; LPS, lipopolysaccharides; MET, metabolic equivalent of task.
tended to have higher serum levels of LPS compared to patients with smaller, tubular adenoma, and adenoma without high-grade dysplasia, respectively ($P<0.10$). However, the LPS levels did not differ by the number of adenoma ($P = 0.57$, data not shown).

**Discussion**

This small-scale case-control study showed that LPS levels were slightly higher in cases than controls, and slightly higher in ACA that was larger, with villous component, or with high-grade dysplasia than the small or tubular ACA. However, there was no statistically significant association between LPS and the risk of ACA. Larger studies are warranted to further investigate the association between the systemic levels of LPS and risk of colorectal tumors and understand the relevant clinical implications.

It is biologically plausible that LPS can contribute to colorectal carcinogenesis. LPS is classified as a pathogen-associated molecular pattern, which is recognized by pattern recognition receptors, such as toll-like receptors (TLRs), on enterocytes or other immune cells. LPS enter the intestinal capillaries after the death of Gram-negative bacteria in the gut. An animal study showed that an increase in physiological concentrations of LPS led to increased permeability at the tight junction in cells. The increased permeability further caused increased circulating levels of LPS, allowing for further inflammation because the lipid A components of the LPS structure bound to the TLR-4/cluster of differentiation complex on innate immune cells in the circulation, and trigger the production of inflammatory cytokines. This signaling pathway can lead to immune escape, uncontrolled tumor growth, and increased proliferation/metastasis of colorectal carcinoma.

In a European population, a positive association between elevated IgA and IgG anti-LPS immunoglobulins and an increased risk of developing CRC was shown. Another study of 138 cases and 324 controls found that higher concentrations of LPS as measured by the LAL assay were significantly associated with colorectal tubular adenomas. A case-control study in Korea, also used the LAL assay, found that 74 patients with colorectal polyps had higher levels of endotoxin than 71 controls (0.108 vs. 0.049 EU/mL, $P < 0.001$). However, we did not find a significant difference in the serum levels of LPS between ACA and polyp-free controls. Nevertheless, the LSP levels were higher in ACA cases, in patients harboring larger ACA or highly malignant ACA. Whether LPS can serve as a biomarker for colorectal tumor needs further investigation.

We showed that LPS can be detected in individuals without polyps after controlling for the possibility of endotoxin contamination to the best of our ability. To date, the physiological range of LPS has not been well established in healthy individuals. The clinical significance of systemic LPS is elusive. One study evaluated 38 studies that measured LPS levels in the general population. Among the 19 studies that reported an LPS level of EU/mL, the median level was 0.32 EU/mL, and the 25–75% interquartile range was 0.23–3.89 EU/mL. The median LPS value in poly-free participants (0.24 EU/mL) was in the range reported by these previous studies. Our study showed that the kinetic LAL assay can be used to test LPS in human serum in an epidemiologic study. The adequate dilution of the samples was critical in obtaining reproducible measurements.

We included obesity, smoking status, and alcohol as the confounding factors in the statistical model because they are known risk factors for CRC. Smoking was also a significant risk factor of ACA in our study. However, we did not observe an association between smoking status and LPS. While cigarettes do contain LPS and increased levels of lipopolysaccharide-binding protein are found in smokers, the association between serum levels of LPS and cigarette smoking is still unclear. Studies have also shown that excessive alcohol intake is significantly associated with elevated levels of serum LPS, as well as a possible link between obesity and increased serum LPS. We did not observe the association between serum levels of LPS and these host and environmental factors in our small-scale study.

Our study had several strengths. We used glass vials to store the
samples to prevent the possibility of the attachment of LPS to the plastic tubes. The glass vials were baked to prevent the contamination of endotoxin in the environment. We assayed the matched cases and controls in the same experiment batch to avoid the potential batch effect. We used the same lot of assay kit in the analysis to reduce the kit variation. In addition, those samples that had high CV were not included in the final analysis. Participants were excluded if they had a history of cancer, familial adenomatous polyposis, Lynch Syndrome, acute infectious disease, and antibiotic treatment in the past 3 months. Nevertheless, several limitations should be noted. The small sample size (43 paired samples) was the major limitation of the study. A larger sample size would have increased the statistical power of the data analysis. Because we enrolled the study participants from an elective colonoscopy clinic, the pool of the polyp-free controls was relatively small. Lastly, the study population consisted of only male veterans who may not represent women and the general population.

**Future directions**

A larger study is needed to determine whether LPS can be used as a biomarker of microbial translocation or leaky gut in population-based studies on colorectal polyp, benign adenoma, and adenoma with malignant potential. To date, the LPS assay has not been widely used in cancer epidemiologic research. Its clinical value should be explored further. Additional biomarkers can be incorporated to advance our understanding of the inflammatory nature of ACA. Finally, it has been shown that not all the components of LPS stimulate an immune response. Precise detection of the effective component of LPS may have greater clinical relevance.

**Conclusions**

Taken together, our study moves the field forward by showing that LPS can be detected in the serum of polyp-free individuals. We observed a nonsignificantly positive association between serum levels of LPS and ACA. The association between LPS and the risk of colorectal tumor should be further examined in larger studies using the same research approach established in the present study.

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**Conflict of interest**

The authors have no conflict of interests related to this publication.

**Author contributions**

Study concept and design (LJ), acquisition of data (NAB, ARO, DR, DYG, RER, HBE, LJ), analysis and interpretation of data (EC, AK, ARO, DLW, DYG, HBE, LJ), drafting of the manuscript (EC, AK, LJ), critical revision of the manuscript for important intellectual content (ARO, DYG, DLW, HBE), administrative, technical, or material support, study supervision (RER, HBE, LJ).

**References**


Chen E. et al.: LPS and colorectal adenoma
HIV-1 Drug Resistance and Genetic Diversity among Vertically Infected Cameroonian Children and Adolescents

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Abstract

Background and objective: HIV-1 vertically infected children stand a high risk of HIV-1 drug resistance (HIVDR), especially after failure to prevention of mother to child transmission (PMTCT) and pediatric antiretroviral therapy (ART). Thus, surveillance of HIVDR might contribute in delineating optimal pediatric regimens. The objective of this study was to evaluate HIVDR and subtype distribution among ART-naïve and ART-failing children.

Methods: A study was conducted throughout 2017 amongst 102 children/adolescents at the “Chantal BIYA International Reference Centre” (CIRCB) in Cameroon. HIVDR testing was performed in protease-reverse transcriptase (RT) region and interpreted using the Stanford HIVdbv8.5; subtyping was performed using MEGA v7.0.26; and data were analyzed using Epi-info v7.1.3.3, with p < 0.05 considered statistically significant.

Results: Sequences were generated from 63 participants (19 ART-naïve, 44 ART-failure); the median-age was respectively 6 [IQR:3.5–11] and 144 [IQR:116.25–185] months for ART-naïve and ART-failing (median ART-duration: 23.55 [IQR:7.61–60.91] months, 63.6% receiving non-nucleoside RT inhibitors [NNRTI]-based regimens). Among ART-naïve children, overall-HIVDR was 52.6% (10/19), with 31.6% (6/19) to NNRTI, 26.3% (5/19) to nucleoside RT inhibitors (NRTI) and 15.8% (3/19) to ritonavir-boosted protease inhibitor (PI/r). Among ART-failing children, overall-HIVDR was 97.7% (43/44), with 95.4% (42/44) to NNRTI, 90.9% (40/44) to NRTI and 18.2% (8/44) to PI/r. Multi-drug resistance was found in 21.05% (4/19) ART-naïve versus 85.7% (37/44) ART-failing children.

Conclusions: The high rates of HIVDR, in both ART-naive and ART-failing children, particularly in ART-failure, suggest the need for implementation of optimal pediatric ART regimens to prevent high HIVDR.
ive and ART-failing children, suggest using genotypic HIV-1 drug resistance testing for selecting optimal pediatric ART-regimens. Multi-drug resistance is concerning among children failing ART and prompts the need of new drugs (integrate inhibitors, darunavir/ritonavir) for optimal pediatric ART management.

Introduction

In spite of the 70% decline in the overall incidence of pediatric HIV infections between 2000 and 2015, an estimated 180,000 children became infected worldwide in 2017, giving a total of 1.8 million children (<15 years old) living with HIV globally, of whom 90% live in sub-Saharan Africa (SSA).1-3 Even though there is progress in the universal coverage of pediatric antiretroviral therapy (ART) in SSA, risks of pediatric HIV-associated mortality remain concerning in this setting due to, but not limited to, delayed initiation and suboptimal monitoring of ART, added to the known high viral load, the immunological immaturity at early age and rapid disease progression among infected children.4-6

Though progress in the HIV prevention of mother to child transmission (PMTCT) services, ranging from options A (i.e. azidothymidine [AZT]), B (i.e. ART during pregnancy and breastfeeding) to the current B+ (i.e. lifelong ART regardless of clinical and immunological status), represents the cornerstone in eliminating new cases of pediatric infections, 4-7,9 rates of HIV vertical transmission are still beyond five percent in several PMTCT high priority countries, including Cameroon.4-7 Furthermore, risks of both pretreatment and acquired HIV drug resistance (HIVDR) become more threatening for every infected child in the frame of failure to current PMTCT strategy and pediatric ART services respectively.8-10 Of note, one in two (50%) infected children harbored pretreatment HIVDR and treated children experience early ART failure with acquired HIVDR.9 Thus, pediatric HIVDR may represent a major hurdle in achieving the third pillar of the 90-90-90 targets in children.10 This hypothesis is plausible in West and Central Africa (WCA) where a total of 2.4 million people, including PMTCT-attendees, are receiving ART (i.e. 40% [25–55%] coverage) and the overall viral suppression rate is still around 70%, with lower outcomes in the pediatric populations.4-10

As in several WCA countries, the therapeutic management of pediatric HIV in Cameroon follows guidelines from the World Health Organization (WHO).11 Of note, initial (first-line) ART depends on PMTCT-exposure: either (a) a ritonavir-boosted protease-inhibitor (PI)-based for PMTCT-exposed HIV-infected children below three years of age, or (b) non-nucleoside reverse-transcriptase inhibitor (NNRTI)-based regimens for peers without PMTCT-exposure and for children aged three years and above (regardless of PMTCT-exposure). In the advent of ART failure, two consecutive viral loads of at least 1,000 RNA copies/mL, therapeutic ART switch is recommended without referring to genotyping for optimal ART selection.8,10-12-14 Thus, in a context with evolving PMTCT interventions, poor adherence to ART and limited access to ART monitoring, suboptimal therapeutic response might be unwavering in sustaining the risks of HIVDR among children initiating or failing ART.15,16

In Cameroon, the rate of HIV MTCT is still beyond 5%17; ART coverage in children is still below 50%,18 viral suppression is around 60–80% for children and slightly above 50% for adolescents.19-28 Prior to wide scale-up of PMTCT, pretreatment HIVDR was low (4.9%) while acquired HIVDR was very high (90%) following ART failure at a median of two years in this country.21,22 After the wide coverage of PMTCT, the increasing access to ART for children and adolescents and the fast growing genetic diversity of HIV-1 in the country, it is then crucial to set-up bold and innovative approaches in the pediatric AIDS response tailored to the specific needs of the local epidemic.23

With the goal to generate new findings, in the era of option B+, towards informed decision-making for more efficient ART strategies and therapeutic monitoring in children, we sought to determine the rates of pre-treatment HIVDR (PDR), acquired HIVDR (ADR) and the HIV-genetic variability among vertically infected Cameroonian children.

Materials and methods

Study design, settings and population

A cross-sectional and analytical study was conducted throughout the year 2017 in a population of 102 HIV-infected children and adolescents (33 ART-naïve and 69 ART-experienced) from four regions of Cameroon: Center, Littoral, West and North-West regions. Laboratory analyses for HIV-1 early-infant diagnosis (EID), viral load measurement, CD4 T cells enumeration and HIVDR testing were carried out at the Chantal BIYA International Reference Centre for HIV/AIDS prevention and management (CIRCB), located in Yaoundé, Cameroon.

The CIRCB is a government institution of the Ministry of Public Health dedicated to HIV research and patient monitoring in several aspects, among which: (a) HIV early infant diagnosis in the frame of the national PMTCT program; (b) diagnosis of co-infections with HIV; (c) viral load measurement; (d) CD4 and CD8 T lymphocytes counts; (e) biochemical and hematological tests for drug safety; (f) genotypic HIVDR testing (GRT) at subsidized costs; with quality control programs conducted in partnership with Quality Assessment and Standardization of Indicators (QASI) and other international agencies (http://www.circb.cm/btc_circb/web/).

Ethical considerations

Administrative authorizations were issued by the CIRCB and the Ministry of Public Health (N°2014/05/631); ethical clearance for the study was obtained from the National Ethics Committee for research on human health (Authorization Number: N°2017/03/877/CNERSH/SP); written informed consent for each participant was obtained from parents or legal guardians; data were protected by the use of specific identifiers for purpose of confidentiality and stored in a password encrypted computer; all laboratory results were freely returned to participants for benefit in their personal clinical management.

Enrolment of study participants

On one hand, infants from the aforementioned clinical sites, referred to CIRCB for HIV-1 EID and whose result was HIV positive, were included in the PDR study arm. On the other hand, ART-experienced children, with a suspicion of ART failure from one of the clinical sites, were also referred to CIRCB for GRT in case of a

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HIV drug resistance and subtypes confirmed virological failure (VF).

**CD4 cell count and viral load measurement**

CD4 cell count was performed using the BD FACs Count system as per the manufacturer’s instructions (https://www.bdbiosciences.com/documents/BD_FACSCount_Brochure.pdf).

HIV-1 RNA quantification was performed on plasma samples using the Abbott m2000rt Real Time HIV-platform (Abbott Molecular Inc. 1300 E. Touhy Ave. Des Plaines, IL 60018 200680-105; USA) according to manufacturer instructions (www.abbottmolecular.com/products/infectious-diseases/realtime-pcr/hiv-1 assay). Briefly, a protocol using 0.6 mL of plasma was used for RNA extraction, followed by a simultaneous amplification and detection on a real-time polymerase chain reaction (RT-PCR). The lower and upper detection threshold of the assay was respectively <40 and >10,000 HIV-1 RNA copies/mL.

**HIV-1 genotypic drug resistance testing**

HIV-1 GRT was performed on plasma samples following an in-house protease-reverse transcriptase genotyping assay. Briefly, RNA was extracted after concentration from 1 mL of plasma aliquots using the QI Amp Viral RNA mini kit (Qiagen, Milan, Italy), according to the manufacturer’s protocol. RNA was then retrotranscribed and amplified using the kit One-Step Invitrogen (Foster City, CA) (SuperScript One-Step for long templates RT-PCR) and 2 different sequence-specific primers [5′-GAC AGG CTA ATT TTT TAG GG-3′ (2075-2094) and 5′ -GAT AAA GGA AAA GGA AGG-3′ (2660-2683)] for 40 cycles. A second round PCR (semi-nested PCR) was performed with another set of two primers for 35 cycles [5′- GAC AGG CTA ATT TTT TAG GG-3′ (2075-2094) and 5′-CCT TGT TTC TGT ATT GCT-3′ (3527-3547) pol]. After PCR products purification using Amicon kits, a direct Sanger-sequencing reaction was then performed using 8 overlapping primers [5′-GAG ATG CAA GGA GAA AAA GGA AGG-3′ (2660-2683 pol), 5′-CCC TTT GAC ACC ATG TTG-3′ (2985-3004), with an insertion, 5′-GCT TCC ACA GGG ATG GAA A-3′ (2993-3011 pol), 5′-CTG ATT TTT AGT ATG CAT ATG ATG-3′ (3504-3527 pol), 5′-CCT TGT TCC ATG TAT CTT GCT TCT GCT-3′ (3527-3547 pol)] and the final product was purified using Sephadex G-50 fine powder.

**HIV-1 sequence analysis**

Sequence data were obtained after capillary electrophoresis on a Genetic Analyzer (Applied Biosystems 3500; Life Sciences, Foster City, CA; 08 capillaries), and complete sequences encompassing the pol region of interest (~1,297 nucleotides) and corresponding to the entire protease region and the first 300 amino acids of the reverse transcriptase open reading frame) were assembled and manually edited using Seqscape software v2.7 (Applied Biosystems, Foster city, CA).

**HIV drug resistance mutations and interpretation**

Sequences generated were analyzed for DR mutations (DRMs) using the Stanford University HIV Drug Resistance Database (http://www.hivdb.stanford.edu) and reported separately for ART-naïve and ART-failing children. In each study group, frequency and types of HIVDR mutations were reported. Sequences having a mixture of wild-type and mutant residues at single drug-resistance-associated positions were also considered to have the mutants at those positions. The nucleotide sequences from this study are submitted to a public repository under the following accession numbers: GenBank MK867695-MK867757.

**Phylogeny analysis**

All the generated sequences were aligned in BioEdit version 7.2.6 (Tom Hall, Raleigh, NC) using CLUSTAL W, and compared with reference sequences for the major HIV-1 subtypes and circulating recombinant forms (CRFs), available in the Los Alamos database (http://www.hiv.lanl.gov), gaps were then removed from the final alignment. The phylogenetic tree was inferred using Maximum likelihood method on the MEGA software v7.0.26 for both subtyping and to ensure that there was no cross-contamination of samples. The statistical robustness and reliability of the branching order within the phylogenetic tree were confirmed through a bootstrap analysis using 1,000 replicates on a maximum likelihood tree obtained by molecular phylogeny. Recombination among HIV-1 clades was assessed by HIV BLAST (https://www.hiv.lanl.gov/content/sequence/BLAST/blast.html), REGA v3 (REGA Institute, KU Leuven, Belgium), COMET (https://comet. lih.lu/index.php?cat=hiv1), RDP v48 (Oxford, England) and SplitTree4 v.4.14.6 (Tuebingen University, Germany; Otago University, New Zealand).

**Statistical analysis**

Data were analyzed using the Epi-info v7.1.3.3, Microsoft Access and Excel 2013. Parametric and non-parametric tests were used for both univariate and multivariate analysis. Fisher exact test, Chi square test and Spearman correlation were used to describe the associations between our variables with p-values <0.05 considered statistically significant.

**Results**

**Sequencing performance and study enrollees**

Out of the 102 children/adolescents enrolled in the study, low-level viremia (<3Log RNA copies/mL) was found from 33 samples, likely due to either prophylactic effect of PMTCT-exposure (n = 13) or the therapeutic effect of ART (n = 20). Of the 69 patients with eligible viremia for sequencing (≥3Log RNA copies/mL), 63 completed sequences were generated, giving a sequencing performance of 91.3%, as shown in the flow chart (Fig. 1). Thus, only the 63 patients with available sequences (accession numbers MK867695-MK867757) were included as study participants in the final dataset for analysis.

**Characteristics of study participants**

Of the 63 study participants considered for analysis, the sex distribution was similar (52.4% boys and 47.6% girls) and their age...
ranged from 2 to 239 months. According to exposure to highly active ART (HAART), 19 children were naïve to HAART and 44 were experiencing VF following exposure to HAART.

Among HAART-naïve participants (n = 19), the median age [interquartile range, IQR] was 6 [3.5–11] months; the CD4 count ranged from 12–2,897 cells/mm$^3$ and the viral load ranged from 3,855–10,000,000 HIV-1 RNA copies/mL. According to PMTCT prophylaxis, seven were reported not to have being exposed to antiretrovirals (ARV), while 12 were reported exposed to ARV prophylaxis of which nine exposed to single dose nevirapine (sd-NVP), two exposed to AZT and one exposed to both NVP and AZT (see Table 1).

Among participants on ART experiencing VF, the median age [IQR] was 144 [116.25–185] months; CD4 count ranged from 2–3,436 cells/mm$^3$ and the plasma viral load from 1,280–6,664,000 copies/mL. The median duration on ART [IQR] was 23.55 [7.61–60.91] months, with 63.6% (28/44) receiving an NNRTI-based regimen versus 36.4% (16/44) receiving a PI/r-based regimen.

### Pre-treatment HIV-I drug resistance in the study population

Among HAART-naïve participants, the overall threshold of PDR was 52.6% (10/19). Considering reports of PMTCT-exposure, 50% (6/12) of participants reported to be exposed to ARV for PMTCT prophylaxis harbored PDR mutations while 57.14% (4/7) of those reported without any ARV exposure harbored PDR; without any statistically significant difference between the two groups (p = 0.57).

The rate of PDR varied by ARV drug class, ranging from 31.6% (6/19), 26.3% (5/19) and 15.8% (3/19) resistance to NNRTI, NRTI to PI/r respectively; which were all far above the WHO’s threshold of 10% PDR (Fig. 2). Interestingly, multi-drug resistance was observed in 21.05% (4/19) of participants: two being resistant to both NRTI and NNRTI, one to both NRTI and PI/r, and one to both NNRTI and PI/r.

Of the six participants harboring major NNRTI-DRMs, mutation Y181C was the most frequent (5/6) in RT-region, conferring high levels of resistance to both first and second generation NNRTIs. Of the five participants with major NRTI-DRMs, L74V/I was the most frequent (2/5) in the RT-region, conferring resistance to abacavir (ABC) and in lesser extend to tenofovir (TDF). Of the three participants with PI/r-DRMs, equal frequencies (1/3) of the mutations M46I, V32I, and D30N were found in the PR-region, conferring intermediate levels of resistance to ritonavir boosted lopinavir, ritonavir boosted atazanavir and even ritonavir boosted darunavir (DRV/r) to certain extent.

### Acquired HIV drug resistance in the study population

Among participants experiencing VF, the overall rate of ADR was 97.7% (43/44), stratified by drug class as follows: 95.4% (42/44), 90.9% (40/44) and 18.2% (8/44) resistance respectively to NNRTI, NRTI and PI/r. Regarding multi-drug resistance, dual-class of ADR (NNRTI and NRTI) was 85.71% (24/28) versus 93.75% (15/16) among those on first-line versus second-line ART (p =...
0.638); while triple class of ADR (NNRTI, NRTI and PI/r) was 0% (0/28) versus 50% (8/16) respectively among those on first-line versus second-line ART (p < 0.0001).

Among the 42 participants harboring NNRTI-DRMs, K103NS (19/42) and Y181C (16/42) mutations were the most frequent; among the 40 participants harboring NRTI-DRMs, M184VI was the most frequent (34/40), followed by Thymidine Analogue Mutations (T215YF [25/40], and M41L [18/40]); among the eight participants with PI/r-DRMs, M46IV (6/8), V82A (3/8) and L76V (3/8) mutations were the most frequent.

### Potential efficacy of antiretroviral drugs for clinical management

According to ART-regimens and based on the genotypic susceptibility scores, the predictive efficacies of drug class ranged from 57.89% for NNRTIs to 89.47% for PI/r among HAART-naïve participants; from 0.00% for NNRTIs to 100.00% for PI/r among those failing first-line ART; and from 0.00% for NNRTIs to 50.00% for PI/r among those failing second-line ART (Fig. 3). Of note, only those failing second-line had a considerable reduced activity of PI/r (50%), with about 18.75% (3/16) showing a reduced activity of DRV/r.

### HIV-1 genetic diversity in the study population

In the entire study population, 13 different viral strains were found (seven in the populations of HAART-naïve and eight in the population of ART-failure), with a prevailing circulating recombinant form (CRF), CRF02_AG (68.2%), followed by G (9.5%), F2 (4.8%) and other subtypes (17.5%), as shown in Figure 4.

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**Table 1. Description of PMTCT prophylaxis in HAART-naïve population**

<table>
<thead>
<tr>
<th>Age of the child (in months)</th>
<th>ARV prophylaxis</th>
<th>Duration of prophylaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Sd-NVP</td>
<td>6 weeks</td>
</tr>
<tr>
<td>2</td>
<td>Sd-NVP</td>
<td>6 weeks</td>
</tr>
<tr>
<td>3</td>
<td>Sd-NVP</td>
<td>6 weeks</td>
</tr>
<tr>
<td>4</td>
<td>Sd-NVP</td>
<td>6 weeks (as from week 3)</td>
</tr>
<tr>
<td>5</td>
<td>AZT</td>
<td>6 weeks</td>
</tr>
<tr>
<td>6</td>
<td>Sd-NVP</td>
<td>6 weeks</td>
</tr>
<tr>
<td>7</td>
<td>Sd-NVP and then AZT</td>
<td>6 weeks</td>
</tr>
<tr>
<td>8</td>
<td>Sd-NVP</td>
<td>6 weeks</td>
</tr>
<tr>
<td>9</td>
<td>AZT</td>
<td>6 weeks</td>
</tr>
<tr>
<td>26</td>
<td>Sd-NVP</td>
<td>6 weeks (as from month 3)</td>
</tr>
<tr>
<td>169</td>
<td>Sd-NVP</td>
<td>6 weeks</td>
</tr>
</tbody>
</table>

ARV, antiretroviral; AZT, azidothymidine; HAART, highly active antiretroviral therapy; PMTCT, prevention of mother-to-child transmission of HIV; Sd-NVP, single dose nevirapine.

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**Fig. 2. Distribution of pre-treatment HIV drug resistance according to PMTCT-exposure.** Horizontal line at 10% indicates the threshold established by the World Health Organization to consider high rate of pre-treatment drug resistance. HIVDR, HIV drug resistance; NNRTI, non-nucleoside reverse transcriptase inhibitors; DRM, drug resistance mutation; PI/r, ritonavir-boosted protease inhibitor.
The genetic diversity had no major effect on the presence of DRMs (p = 0.99). Table 2 shows the similar distribution of ADR between 02_AG versus non-AG infected participants.

**Discussion**

As pediatric HIV infection tends to decrease overtime, follow-

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**Table 2. Effect of HIV-1 genetic diversity on acquired drug resistance**

<table>
<thead>
<tr>
<th>Rates of DRMs</th>
<th>CRF02_AG</th>
<th>Non-CRF02_AG</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall rate</td>
<td>96.88% (31/32)</td>
<td>100% (12/12)</td>
<td>0.53</td>
</tr>
<tr>
<td>NNRTI-DRMs</td>
<td>93.75% (30/32)</td>
<td>100% (12/12)</td>
<td>0.52</td>
</tr>
<tr>
<td>NRTI-DRMs</td>
<td>86.63% (29/32)</td>
<td>91.67% (11/12)</td>
<td>0.70</td>
</tr>
<tr>
<td>PI-DRMs</td>
<td>15.63% (5/32)</td>
<td>25% (3/12)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

CRF, circulating recombinant form; DRMs, drug resistance mutations; HIV-1, human immunodeficiency virus type 1; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitors.

Fig. 3. Level of predictive effectiveness of different ARV drug classes. NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitor.

Fig. 4. HIV-1 phylogenetic tree of the 63 perinatally infected participants. The reference sequences were from the Los Alamos Database (https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html); some references have been omitted to enable better visualization. The scale bar represents 1% genetic distance. CRF, circulating recombinant form.
mainly attributed to the scale of PMTCT interventions. Thus, with increasing access to option B+, PDR would be present in more than half of HIV-positive infants, especially with the use of next generation sequencing platforms. 

Among children failing ART, treatment failure occurred about two years after therapeutic initiation (ranging between one to five years for the majority). This confirms the event of early ART failure among children and the need for timeous viral load monitoring, as previously reported in the several settings of the same country.14,22,29 

The high burden of ADR among children failing the first- and second-line regimens reveal almost complete inefficacy of NNRTIs and NRTIs, which are low genetic barrier drugs, contributing to early selection of DRMs among children, especially in the frame of poor compliance to ART regularly reported among children and adolescents.15,29,34 Interestingly, dual-class of resistance to NNRTI and NRTI remains very high (>80%) among children failing first- and second-line ART, with very low rate resistance to PI/r. This underscores the benefit of using PI/r-based regimens or other NNRTI-sparing ART combinations as initial or first-line ART among children diagnosed HIV positive.33 Of relevance, 50% of children on second-line have DRMs to PI/r, indicating that about half of children experiencing VF on PI/r-based second line are in need of third-line ART regimens containing integrase strand-transfer inhibitors (dolutegravir, raltegravir) and/or DRV/r.35,36 However, with about one fifth risk of reduced efficacy of DRV/r (~19% in our study) following exposure to the classical PI/r-based regimen, alongside the well documented high risk of inactive RTIs,36 close viral load testing and GRT should be strongly recommended prior to switching to third-line ART even in children and adolescents.36-38

Despite the broad viral genetic diversity among Cameroonian populations,39,40 there was no considerable effect of the major circulating subtype (i.e. CRF02_AG) on emerging DRMs.39,31 Therefore, although HIV variability may be associated to viral fitness, disease progression and drug susceptibility,41 our findings reveal that subtype disparity might not have a major clinical relevance in the Cameroonian pediatric context at the moment.34,36

Our study has some limitations. Firstly, the limited sample size in HAART-naive children diagnosed HIV-positive in our EID program (due to declining vertical transmission with PMTCT success and treat all implementation) restricted the breath of PDR appraisal and the true effect of PMTCT (due to recall bias). Thus, clinicians should not focus on maternal recall on PMTCT-exposure in guiding pediatric ART regimens.38 Furthermore, testing for drug concentration (not performed in our study) for uncertain exposure to PMTCT would help in screening children with potential wild type viruses at ART initiation.

Future directions

For a better understanding of the impact of PDR in pediatric populations, further investigations will focus on assessing drug resistance patterns in a larger population, considering the selection of mutations according to HIV-1 subtypes/recombinants, maternal ART history and feeding options throughout the PMTCT cascade care. Assessing the effect of infant age on the trends of PDR would also help in timing pediatric ART regimen. Lastly, surveys of acquired HIVDR to newer drugs will help in tailoring future optimal second/third-line pediatric regimens.

Conclusions

In a nutshell, HIVDR appeared very high among Cameroonian children. The high burden of PDR detected at infancy could be due to scale-up in PMTCT. The early VF (~24 months of pediatric-ART) was associated to the presence of ADR, with multidrug resistance to RTIs that requires innovative drugs (integrase strand transfer inhibitor, preferably and DRV/r). This advocates for GRT to guide initial ART and to select for optimal ART regimens when switching to subsequent regimens in children. The broad HIV-1 genetic variability does not appear with any potential clinical relevance on the emergence of DRMs.

Acknowledgments

We are appreciative to our institutional staff (CIRCB) who participated in the enrolment and in sample processing. We are thankful to the children and adolescents, their parents or guardian for their consents. We also acknowledge Ghylaine Bruna Djeunang Dongho, Armand Tiotsia Tsapi and Irenée Domkam for their comments and remarks in the data analysis and interpretation of findings.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived the study and designed the experiment (BD, JF, ESN), acquired the data (BD, JF, ESN, DT, GT), analyzed and interpreted the data (BD, JF, ESN, LCMM, NK, SMS, SCB, AEN, MSS, CN, PNK, FN, VC, CFP, AN); drafting of the manuscript (BD, JF, ESN), critical revision of the manuscript for important intellectual content (DT, MSS, GT, GB, LCMM), study supervision (CN, Dambaya B. et al: HIV drug resistance and subtypes

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Dambaya B. et al: HIV drug resistance and subtypes


Original Article

Neonatal Near Miss and Its Associated Factors at Injibara General Hospital, Awi Zone, Northwest Ethiopia, 2019

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Abstract

Background and objectives: There are many newborns who suffer a life-threatening complication in many low-resource countries. Neonatal near miss has been proposed as a tool to evaluate and improve the quality of neonatal care. However, there has been limited evidence on magnitude of neonatal near miss and determinant factors in Ethiopia. The aim of this study was to assess proportion and associated factors of neonatal near miss among neonates delivered at Injibara General Hospital, Awi Zone, Northwest Ethiopia, 2019.

Methods: This institutional-based cross-sectional study was conducted from February 1, 2019 to April 30, 2019, among 404 neonates. A structured and pretested questionnaire was used for mothers and a standard checklist was used for their neonates. Bivariate and multivariate logistic regression modelings were fitted to identify factors associated with neonatal near miss. An adjusted odds ratio (AOR) with 95% confidence interval (CI) was computed to determine the level of significance.

Results: The proportion of neonatal near miss was found to be 23.3% with 95% CI of 19.1–27.7%. Primiparous (AOR: 2.01, 95% CI: 1.03–3.95), referral linkage (AOR: 3.23, 95% CI: 1.89–5.513), maternal perception of reduced fetal movement (AOR: 5.95, 95% CI: 2.47–14.33), premature rupture of membrane (AOR: 3.10, 95% CI: 1.27–5.59), prolonged labor (AOR: 3.00, 95% CI: 1.28–7.06), obstructed labor/cephalo-pelvic disproportion (AOR: 4.05, 95% CI: 1.55–10.57), and non-reassuring fetal heart rate pattern (AOR: 3.75, 95% CI: 1.69–8.33) were significantly associated with neonatal near miss.

Conclusions: The proportion of neonatal near miss in the study area was found to be higher than that found by the World Health Organization’s neonatal near miss systemic review. Strengthened referral linkage and efforts is needed to avoid preventable causes of neonatal morbidity and mortality.

Keywords: Neonatal Near Miss; Proportion; Awi Zone; Ethiopia.

Abbreviations: AOR, adjusted odds ratio; APGAR, appearance, pulse, grimace, activity and respiration; CI, confidence interval; EDHS, Ethiopia Demographic and Health Survey; NNM, neonatal near miss; WHO, World Health Organization.

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of antenatal steroid, use of parenteral nutrition and identification of congenital malformation.\textsuperscript{2,4,5}

The near miss concept is a potentially useful approach to assess quality of newborn care but due to the enormous variability of socio-demographic and technological advances in newborn care and registration of health information, there is no consensus on establishment of criteria of NNM.\textsuperscript{6,7} Globally, 2.5 million newborns died in the first month of life in 2017. The majority of all neonatal deaths (75%) occur during the first week of life and about 1 million newborns die within the first 24 hours.\textsuperscript{8} The NNM rates in different studies have included 220/1,000 live births in Northeastern Brazil, 39.2/1,000 live births in the ‘Birth in Brazil’ survey, and 72.5/1,000 live births in the World Health Organization’s (WHO’s) multicountry survey.\textsuperscript{7,9,10}

Despite a decrease in the neonatal mortality rate, particularly in low and middle-income countries, the neonatal morbidity rate remains elevated. It is estimated that the number of survivors from a NNM event is three- to six-times higher than the number of neonatal deaths.\textsuperscript{3,11,12} Even though the causes of neonatal morbidity and mortality are preventable and treatable, and the fact that low-cost and effective interventions exist, there has been no significant decline in neonatal morbidity and mortality.\textsuperscript{13}

According to the Ethiopia Demographic and Health Survey (commonly referred to as the EDHS) 2016, neonatal mortality declined from 49 deaths per 1,000 live births in 2000 to 29–36 deaths per 1,000 births in 2016, a reduction of 41% over the past 16 years.\textsuperscript{14} In 2019, the Ethiopian Mini Demographic and Health Survey reported the neonatal mortality rate to be 30 deaths per 1,000 live births. While both numbers and rates have been decreasing over the last 16 years, they have remained stable since the 2016 EDHS.\textsuperscript{15}

Even though Ethiopia established priorities to reduce under five years mortality, such as by increasing skilled birth attendants present at a birth, meeting the unmet need of family planning, improving quality of care, increasing community-based newborn care and expanding quality facilities for newborn care, increasing resources for health financing and increasing focus on research and innovation, the improvement of neonatal morbidity and mortality remains an unaccomplished agenda.\textsuperscript{16}

There is limited evidence regarding the NNM concept in Ethiopia. Moreover, available studies are focused on neonatal mortality. Therefore, this study aimed to assess the proportion of and factors associated with NNM among neonates in Ethiopia, using a population of neonates delivered at the Injibara General Hospital in the Awi Zone.

**Methods**

**Study design and setting**

This institutional-based cross-sectional study was conducted among 404 neonates at the Injibara General Hospital, Awi Zone, Amhara Region, Northwest Ethiopia from February 1, 2019 to April 30, 2019. The town of Injibara is the capital of the administrative center of Awi Zone. It is located about 447 km away from the capital city of Ethiopia, Addis Ababa, and 118 km from Bahir Dar, a city of the Amhara National Regional State. The Injibara General Hospital provides health services to more than 1.2 million people and in its catchment area there are 46 health centers and 5 district hospitals. The hospital has different departments that provide outpatient service and inpatient service, and has an operative theater department.

Population

All neonates delivered at Injibara General Hospital were the source population. All neonates delivered at Injibara General Hospital and within the first 28 days of neonatal life from February 1, 2019 to April 30, 2019 were the study population.

Inclusion and exclusion criteria

All neonates with their mothers and within 28 days of neonatal life were eligible to participate, while neonates with gestational age less than 28 completed weeks, readmitted neonates, and congenital anomalous were excluded.

**Ethical approval and consent to participate**

Ethical clearance was obtained from the ethical review board of Bahir Dar University College of Medicine and Health Science and a supporting letter was written to Injibara General Hospital, Awi Zone. Verbal informed consent was obtained from each study participant after being given an explanation of the purpose and objective of the study.

**Sample size determination**

The single-population proportion formula was used to determine sample size. Consideration was made for 50% of live births surviving life-threatening conditions during the neonatal period, at 5% margin of error (w) and 95% \(Z_a/2 = 1.96\) confidence interval (CI). To compensate for non-responses, 5% of determined sample size was added, and the final sample size was 404.

**Sampling technique**

Systematic sampling technique was used to obtain all study subjects. Based on the order of registration on the postnatal log book, data at every other interval was collected from mothers and their neonates until the desired amount of samples were obtained.

**Operational definition**

NNM rate was calculated as \([\text{number of NNM cases/total number of live births}] \times 1,000\). NNM was considered when the newborn faced at least one of the following proposed criteria but survived those complications.

**Pragmatic criteria**

Birth weight <2,500 g, gestational age <37 weeks; 5th-minute AP-

**Management criteria**

Mechanical ventilation, cardiopulmonary resuscitation, intubation, nasal continuous positive airway pressure, parenteral antibiotics, parenteral nutrition, vasoactive drugs, phototherapy during the first
28 days, anticonvulsants, blood products, steroids for the treatment of refractory hypoglycemia, surgical procedures, and antenatal steroids.

**Data collection procedure**

A combination of data collection methods was used. The data from mothers was collected by using pre-tested interviewer-administered structured questionnaire which was adapted from literature reviews; maternal charts were also reviewed, for clarity of diagnosis and intervention. Data from their neonates were collected by using a standardized checklist adapted from different publications of such in the literature, each of which had been developed for similar purpose by different authors. The maternal data collection tool (the questionnaire, see Supplemental Document S1) was prepared first in English, then translated to a local language (Amharic and Agew) and then re-translated back to English to verify the consistency and content of the questionnaire. Data were collected by five Bachelors of Science midwives and supervised by two senior Bachelors of Science midwives.

**Data quality assurance**

Training was given for data collectors and supervisors, regarding to the objectives of the study, method of data collection and significance of the study, to prevent any confusion and have a common understanding about the study. Pre-test was conducted for 10% of the total sample at another district hospital that had similar characteristics with the study population. Throughout the course of the data collection, interviewers was supervised and regular meetings were held between the data collectors, the supervisor and the principal investigator, together, in which problematic issues arising from interviews were discussed and addressed. The collected data were reviewed and checked for completeness before data entry.

**Data analysis**

The collected data were checked, coded and entered into Epi-data (version 3.5) software, then exported into SPSS software (version 20) for analysis. Bivariate analysis was performed for all explanatory variables in relation to NNM. Variables having p-value <0.20 in the bivariate analysis were selected for the multivariate logistic regression modeling, for adjustment of confounding effects between explanatory variables. Adjusted odds ratio (AOR) with 95% CI was computed and variables having p-value <0.05 in the multivariate logistic regression model were considered as statistically significant. Odds ratio was also used to determine the strength of association between independent variables and the outcome variable.

**Results**

**Socio-demographic characteristics of the study subjects in Injibara General Hospital, Awi Zone, Northwest Ethiopia, 2019**

A total of 404 mothers with their neonates was interviewed, with a response rate of 100%. Newborns’ mothers were in the age group of 20–34 years, and 311 (77%) had the mean age of 29.0 years (standard deviation: 5.4 years). The majority of mothers (n = 400, 99%) were currently married and 391 (96.8%) were orthodox religious followers. Nearly three-fourths (n = 289, 71.5%) were Agew ethnicity, and over one-half (n = 254, 63%) were urban residents. Regarding educational status, 150 (37.1%) mothers had not attended formal education, 58 (14.4%) had attended up to receipt of diploma and above. Over one-half (n = 245, 60.7%) of the mothers were housewives (Table 1).

**Obstetrics characteristic of mothers**

All selected mothers of newborns attended at list one ante natal care follow-up visit. Women who had attended one to three ante natal care visit(s) numbered 213 (52.7%) and a high proportion of NNM cases was found in this category of ante natal care follow up visit(s), numbering 57 (60.6%). Twenty-eight percent of mothers were primiparous. A high rate of NNM cases was observed in primiparous mothers (n = 36, 38.3%). A total of 148 (36.6%) mothers of newborns were referred from other health institutions, and among them more than half (n = 56, 60%) represented NNM cases; obstetrics complications during the current pregnancy and labor-delivery were noted for 72 (17.8%) and 89 (22%) of mothers respectively (Table 2).

**NNM characteristic**

A total of 94 (23.3%) live birth neonates met the criteria of NNM. Among the NNM selection criteria, mechanical ventilation was the most commonly identified (n = 50, 53%), with a proportion of 124/1,000 live births, and 35 (37%) of NNM cases represented less than 37 completed weeks of gestation (preterm birth), with a proportion of 86.6/1,000 live births. Almost one-third of NNM cases were low birth weight (n = 29, 31%), accounting for almost 71.8/1,000 live births. More than half of the NNM cases (n = 53, 58%) were faced with more than one NNM criteria (Table 3).

**Factors associated with NNM**

Multivariate logistic regressions revealed that primiparous, referral linkage, premature rupture of membrane, maternal perception of reduced fetal movement, obstructed labor/cephalo-pelvic disproportion, prolonged labor and non-reassuring fetal heart rate pattern were significantly associated with NNM. Women who were primiparous showed two-times increased odds of NNM, as compared to mothers who were grand multiparous (AOR: 2.06, 95% CI: 1.06–3.98). Women who were referred from other health institutions showed three-times increased odds of NNM, as compared to non-referral cases (AOR: 3.23, 95% CI: 1.89–5.51).

Of the obstetric complications faced during the current pregnancy, premature rupture of membrane showed three-times increased odds of NNM (AOR: 3.10, 95% CI: 1.27–7.59). Maternal perception of reduced fetal movement showed almost six-times increased odds of NNM, as compared to their counter parts (AOR: 5.95, 95% CI: 2.47–14.33).

Of the obstetric complications experienced during labor-delivery, women with prolonged labor (>24 hours of labor) showed three-times increased odds of NNM, as compared to delivery within 24 hours of labor (AOR: 3.00; 95% CI: 1.28–7.06). Women with obstructed labor/cephalo-pelvic disproportion showed four-
<table>
<thead>
<tr>
<th>Variables, $n = 404$</th>
<th>Selected mothers with their live births</th>
<th>$n$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td></td>
<td>17 (4.2)</td>
</tr>
<tr>
<td>20–34</td>
<td></td>
<td>311 (77)</td>
</tr>
<tr>
<td>&gt;=35</td>
<td></td>
<td>76 (18.8)</td>
</tr>
<tr>
<td><strong>Marital status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Currently in marital union</td>
<td></td>
<td>400 (99)</td>
</tr>
<tr>
<td>Currently not in marital union</td>
<td></td>
<td>4 (1)</td>
</tr>
<tr>
<td><strong>Religion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orthodox</td>
<td></td>
<td>391 (96.8)</td>
</tr>
<tr>
<td>Muslim</td>
<td></td>
<td>13 (3.2)</td>
</tr>
<tr>
<td><strong>Resident</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td></td>
<td>254 (62.9)</td>
</tr>
<tr>
<td>Rural</td>
<td></td>
<td>150 (37.1)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agew</td>
<td></td>
<td>289 (71.5)</td>
</tr>
<tr>
<td>Amhara</td>
<td></td>
<td>110 (27)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td>5 (1.2)</td>
</tr>
<tr>
<td><strong>Maternal education</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No formal education</td>
<td></td>
<td>150 (37.1)</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td>144 (35.6)</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td>52 (12.9)</td>
</tr>
<tr>
<td>Diploma and above</td>
<td></td>
<td>58 (14.4)</td>
</tr>
<tr>
<td><strong>Partner education</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No formal education</td>
<td></td>
<td>117 (29)</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td>157 (39)</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td>39 (10)</td>
</tr>
<tr>
<td>Diploma and above</td>
<td></td>
<td>87 (22)</td>
</tr>
<tr>
<td><strong>Maternal occupation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Housewife</td>
<td></td>
<td>245 (60.7)</td>
</tr>
<tr>
<td>Governmental employed</td>
<td></td>
<td>68 (16.8)</td>
</tr>
<tr>
<td>Non-governmental employed</td>
<td></td>
<td>13 (3.2)</td>
</tr>
<tr>
<td>Merchant</td>
<td></td>
<td>75 (18.6)</td>
</tr>
<tr>
<td>Daily worker</td>
<td></td>
<td>3 (0.7)</td>
</tr>
<tr>
<td><strong>Paternal occupation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farming</td>
<td></td>
<td>166 (41.4)</td>
</tr>
<tr>
<td>Governmental employed</td>
<td></td>
<td>84 (20.9)</td>
</tr>
<tr>
<td>Non-governmental employed</td>
<td></td>
<td>15 (3.7)</td>
</tr>
<tr>
<td>Merchant</td>
<td></td>
<td>132 (32.7)</td>
</tr>
<tr>
<td>Daily worker</td>
<td></td>
<td>3 (0.7)</td>
</tr>
</tbody>
</table>
### Table 2. Obstetric characteristics of the mothers of newborns at Injibara General Hospital, Awi Zone, Northwest Ethiopia, 2019

<table>
<thead>
<tr>
<th>Variables</th>
<th>Selected mothers with their live births</th>
<th>Neonatal near miss, n = 94</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Antenatal care follow-up</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>213 (52.7)</td>
<td>57 (60.6)</td>
</tr>
<tr>
<td>&gt;=4</td>
<td>191 (47.3)</td>
<td>37 (39.4)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>113 (28)</td>
<td>36 (38.3)</td>
</tr>
<tr>
<td>2–3</td>
<td>155 (38.4)</td>
<td>23 (24.5)</td>
</tr>
<tr>
<td>4–5</td>
<td>96 (23.8)</td>
<td>23 (24.5)</td>
</tr>
<tr>
<td>&gt;=6</td>
<td>40 (9.9)</td>
<td>12 (12.7)</td>
</tr>
<tr>
<td>History of abortion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>45 (11)</td>
<td>9 (9.6)</td>
</tr>
<tr>
<td>No</td>
<td>359 (89)</td>
<td>85 (90.4)</td>
</tr>
<tr>
<td>History of still birth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>22 (5.4)</td>
<td>6 (6.4)</td>
</tr>
<tr>
<td>No</td>
<td>382 (94.5)</td>
<td>88 (93.6)</td>
</tr>
<tr>
<td>History of obstetric complication during pregnancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>72 (17.8)</td>
<td>42 (44.7)</td>
</tr>
<tr>
<td>No</td>
<td>332 (82.2)</td>
<td>52 (55.3)</td>
</tr>
<tr>
<td>History of obstetric complication during labor-delivery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>89 (22)</td>
<td>54 (57.4)</td>
</tr>
<tr>
<td>No</td>
<td>315 (78)</td>
<td>40 (42.6)</td>
</tr>
<tr>
<td>Referral from other health institution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>148 (36.6)</td>
<td>56 (60)</td>
</tr>
<tr>
<td>No</td>
<td>256 (63.4)</td>
<td>38 (40)</td>
</tr>
</tbody>
</table>

### Table 3. Criteria for neonatal near miss among live births at Injibara General Hospital, Awi Zone, Northwest Ethiopia, 2019, n = 404

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Proportion of NNM in each category, n = 94</th>
<th>Proportion of NNM/1,000 live births</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Gestational age &lt;37 completed weeks</td>
<td>35 (37.2)</td>
<td>86.6</td>
</tr>
<tr>
<td>Weight less than 2.5 kg</td>
<td>29 (31)</td>
<td>71.8</td>
</tr>
<tr>
<td>5-minute APGAR score &lt;7</td>
<td>21 (22.3)</td>
<td>51.9</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>50 (53.2)</td>
<td>124</td>
</tr>
<tr>
<td>Cardiopulmonary resuscitation</td>
<td>12 (12.8)</td>
<td>29.7</td>
</tr>
<tr>
<td>Intubation</td>
<td>4 (4.2)</td>
<td>1</td>
</tr>
<tr>
<td>Nasal continuous positive airway pressure</td>
<td>11 (11.7)</td>
<td>27</td>
</tr>
<tr>
<td>Parenteral antibiotics</td>
<td>24 (25.5)</td>
<td>59</td>
</tr>
<tr>
<td>Use of parenteral nutrition</td>
<td>13 (13.8)</td>
<td>32</td>
</tr>
<tr>
<td>Vasoactive drugs</td>
<td>5 (5.3)</td>
<td>12.4</td>
</tr>
<tr>
<td>Phototherapy during the first 28 days</td>
<td>2 (2.1)</td>
<td>5</td>
</tr>
<tr>
<td>Anticonvulsants</td>
<td>2 (2.1)</td>
<td>5</td>
</tr>
<tr>
<td>Use of steroids for the treatment of refractory hypoglycemia</td>
<td>3 (3.2)</td>
<td>7.43</td>
</tr>
<tr>
<td>Use antenatal steroid</td>
<td>5 (5.3)</td>
<td>12.4</td>
</tr>
</tbody>
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Note: The values cannot be summed up to 100% because multiple interventions were possible. APGAR, appearance, pulse, grimace, activity and respiration.
times increased odds of NNM, as compared to normal labor (AOR: 4.05; 95% CI: 1.55–10.57). Presence of non-reassuring fetal heart rate pattern showed almost four-times increased odds of NNM, as compared to those who had normal range of fetal heart rate pattern during labor (AOR: 3.75, 95% CI:1.69–8.33) (Table 4).

Discussion

In this study, the proportion of NNM was 23.3%. This finding is consistent with the finding of a study from Northeastern Brazil, which found 22%. This finding is also high compared to a study from the WHO using a multicounty survey (7.25%), of the Birth in Brazil survey (3.92%), of a study in the South of Brazil (3.3%), and a study in Southeastern Brazil (1.7%). The observed variation of NNM rates in a WHO multicounty survey and South-
grand multiparous mothers. This result is in line with the studies in Northeastern Brazil and Southeast Brazil.\textsuperscript{7,23} This also might be due to the fact that primiparous mothers were high-risk for malposition, malpresentation, prolonged labor, increased induction of labor, and obstructed labor/cephalo-pelvic disproportion.

Obstetrics complications during current pregnancy were shown to be statistically significantly associated to NNM. Premature rupture of membrane showed three-times increased odds of NNM as compared to those mothers’ counterparts. This might be due to the fact that premature rupture of membrane usually leads to preterm labor, which is a risk factor for birth asphyxia, choorioamnionitis, neonatal sepsis, pulmonary hypoplasia, and cord prolapse. Different studies revealed that premature rupture of membrane significantly related to increased risk of maternal, fetal and neonatal morbidities and mortalities resulting from obstetric complications.\textsuperscript{24–28}

Women with reduced fetal movement during pregnancy showed almost six-times increased odds of NNM, as compared to mothers who did not perceive reduced fetal movement. This might be due to fetal compromise, utero-placental insufficiency, intrauterine growth restriction, and/or abnormal amniotic fluid volume. Similarly, different studies revealed that reduced fetal movement was associated with poor prenatal outcomes of those with preterm birth, perinatal birth injury, low birth weight, low APGAR score, increase rate of cesarean section, and neonatal and fetal deaths.\textsuperscript{22,23}

Obstetrics complications during labor-delivery were strongly associated with NNM in our study. Women with obstructed labor/cephalo-pelvic disproportion showed four-times increased odds of NNM, as compared to their counterparts. This might be due to obstructed labor causing fetal hypoxia, due to tonic uterine contraction that interferes with the uteroplacental circulation, intracranial hemorrhage due to super molding of the head, birth trauma, and infection. Mothers with prolonged labor showed three-times increased odds of NNM, as compared to mothers who were delivered within 24 hours of labor. This might be due to abnormal progress of labor leading to fetal distress, early neonatal and fetal infections, birth trauma, and fetal hypoxia due to diminished uteroplacental circulation.

Detected non-reassuring fetal heart rate pattern showed almost four-times increased odds of NNM, as compared to detected reassuring fetal heart rate pattern. A supporting study in Indonesia revealed that survival of newborns from mothers without severe complications was better than that of newborns from mothers with obstetric complications, and studies in Brazil also revealed that maternal near miss were strongly associated with prematurity, neonatal asphyxia, and early respiratory discomfort.\textsuperscript{24,25} This finding is also supported by studies from the Jimma University Specialized Hospital and the Dessie Referral Hospital, which showed that obstetric complications during current pregnancy and complications during labor and delivery were strongly associated with adverse birth outcomes (low birth weight, preterm birth, low APGAR score, and still birth).\textsuperscript{26–28}

Referral linkage was found to be significantly associated with NNM in this study, increasing the odds of NNM by three-times as compared to those mothers who faced obstetric complications and needed more timely and better interventions to avoid maternal and neonatal morbidity and mortality.

**Future directions**

It is hypothesized that knowing magnitude and associated factors of NNM is meaningful for stakeholders to intervene on preventable factors, which is also of theoretical and practical significance to researchers, policy makers and practitioners. Based upon the finding of this study, we hypothesized that early identification and treatment of obstetric complications could reduce neonatal morbidity and mortality.

**Conclusions**

The proportion of NNM in the study area of this study was found to be high. Gestational age <37 completed weeks and weight <2.5 kg were the most commonly identified pragmatic criteria, and mechanical ventilation, use of parenteral antibiotics and use of parenteral nutrition were the most commonly identified management criteria. Variables statistically associated with NNM were primiparous, referral linkage, premature rupture of membrane, maternal perception of reduced fetal movement, prolonged labor, obstructed labor/cephalo-pelvic disproportion, and non-reassuring fetal heart rate pattern. Ensuring the continuum of compressive maternal care from pregnancy through delivery will help to avoid preventable causes of neonatal morbidity and mortality and to create good referral linkage with health facilities within catchment areas, including provision of feedback.

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**Data availability**

The data used to support the findings of this study are available from the corresponding author upon formal request.

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**Conflict of interest**

The authors have no conflicts of interest related to this publication.

**Author contributions**

Conceiving the research idea, writing the proposal, designing the study, supervising the data collection process, performing the statistical analysis, and writing the manuscript (HG); participating in the data analysis and revising drafts of the paper (MB); reviewing and finalizing drafts of the paper (SK, TH). All authors read and approved the final manuscript.
Supporting information

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Supplemental Document S1. Research questionnaire.

References


Role of Epigenetic Modification of N6-methyladenosine in Phase Separation

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Abstract

In recent years, the development of biophysical analysis methods has crossed with macromolecular condensates in cells. Researchers are interested in membrane-less organelles assembling into biomolecule ‘aggregates’ with similar liquid-like properties of phase separation. Cell biologists now think that many of the membrane-less organelles observed in cells are formed by phase separation caused by interactions between proteins and nucleic acids. Phase separation, thus, becomes a major player in the control of a variety of biological functions. Nevertheless, the biophysical regulation of these cells is still poorly understood. Here, we reviewed the current literature that collectively reveals the roles of epigenetic modification of N6-methyladenosine (m6A) in phase separation.

Introduction

Phase transition/phase separation of biological macromolecules in cytoplasm has developed rapidly into a hot research area in recent years. Cells consist of many different organelles (or compartments) that separate cell functions. Organelles can be divided into compartments enveloped by lipid membranes (such as the nucleus, vacuoles, endoplasmic reticulum, and mitochondria) and organelles not enveloped by lipid membranes (such as P bodies, nucleoli, pericentric substances, stress granules, and reproductive particles). The distribution and assembly dynamics of non-membrane-bound compartments have aroused great interest over the last ten years and for good reason.1-3 Accumulating evidence has indicated that these non-membrane-bound organelles, such as Cajal bodies, nucleoli, stress granules, synaptic cytoskeleton, and microRNA-induced silencing complex, behave as fluid droplets, which undergo phase separation phenomenon.4,5 Through the study of these non-membrane-bound organelles, scientists have gained a deep understanding of the molecular mechanism of different diseases. Earlier work in this area focused on stress granules involved in cell survival and its relationship with amyotrophic lateral sclerosis. Recently, several studies provided us with fresh insight into the dynamic of RNA modifications and RNA-protein interactions in contributing to phase separation and non-membrane-bound organelles’ formation in cells.6 However, it has never been clarified what triggers the aggregation of some macromolecules rather than others in the same droplet.

Thus, we reviewed the current literature on the roles of the modification of N6-methyladenosine (m6A) in phase separation, with a focus on recent research findings regarding m6A regulation of phase separation.

Keywords: Methylation; Phase separation; m6A; YTHDF protein.


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Fig. 1. m^6A modification is dynamically regulated by writer, eraser, and reader protein. Writer protein is a methyltransferase, like WTAP, METTL3, and METTL14. Eraser protein is a demethylase, like FTO and ALKBHS localized primarily in the nucleus. Reader protein is a binding methylated protein, which is divided into in cytoplasm and in nucleus. In the nucleus, m^6A can be regulated by m^6A readers YTHDC1, HNRNPA2B1, and IGF2BP1/2/3, while in the cytoplasm, m^6A can be regulated by m^6A readers YTHDF1/2/3, YTHDC2, and elf3. WTAP, Wilms tumor 1-associated protein; METTL, methyltransferase like protein; FTO, fat mass and obesity-associated protein; ALKBHS, ALKB homolog 5; YTHDC, YTH domain containing protein; HNRNPA2B1, heterogeneous nuclear ribonucleoprotein A2B1; IGF2BP1/2/3, insulin-like growth factor 2 mRNA binding protein 1/2/3; YTHDF1/2/3, YTH domain family proteins 1/2/3; Prcc2a, proline-rich coiled-coil 2A; HNRNPC, heterogeneous nuclear ribonucleoprotein C; elf3, eukaryotic initiation factor 3; SRSF2, serine and arginine rich splicing factor 2.

The cytoplasm, and the nuclear YTH domain containing protein 1 (YTHDC1) have been identified to be the ‘readers’ of m^6A and to be able to regulate mRNA stability and translation, thereby mediating downstream effects (Fig. 1).^7^9 Recent studies indicated that mRNA metabolism needs the active involvement of the modification of m^6A, in particular modulating mRNA stability, determining cell fate, affecting transcriptional control of cell, and playing an important role in regulating the fate determination of various cell types,^10^ lipid metabolism,^11^ and immunity.^12^ Scientists selected three of the major m^6A-binding YTH domain family proteins YTHDF1,2,3 as research subjects, trying to reveal the regulation mechanism of m^6A on mRNA stability and translation efficiency.^13^ Primary protein sequence analysis has demonstrated that YTHDF protein consists of about 15 kDa of m^6A and a low complexity region of about 40 kDa. It is speculated that the three genes may undergo liquid-liquid phase separation (LLPS) according to this structural arrangement (Fig. 2). YTHDF2 protein was the highest content of YTHDF parologue purified from cells. Several studies have characterized that the factors regulating strength of intermolecular interactions, including temperature, pH and ionic strength, will affect the ultimate phase separation behavior.\(^{15}\) The gene solution of YTHDF2 was transparent at the low-temperature of 4 °C; however, when the temperature raised to 37 °C, the YTHDF2 gene solution became turbid; the solution became clear again at the low-temperature of 4 °C. This finding served to confirm that the YTHDF2 phenomenon was LLPS. Meanwhile, Ries et al.\(^3\) found that YTHDF2 protein at the physiological concentration (about 5 µM) increased sufficiently for phase separation occurring. Those authors additionally examined whether phase separation enhancement of YTHDF2 protein was regulated by m^6A modification in vitro. They confirmed that a 65 nucleotide-long transcriptome regions containing 10 modified m^6A caused a significant increase of the phase separation in YTHDF proteins. Consequently, the authors speculated that adjacent m^6A moieties on RNA promote ‘multivalent interactions’ between the intrinsically disordered regions of YTHDF proteins that were bound to m^6A through their YTH domain, thus facilitating phase separation.

To address whether m^6A can influence the phase separation of YTHDF2 gene, Ries et al.\(^3\) confirmed that endogenous stress granules were formed after exposure to heat shock in m^6A methyltransferase14-knockout mouse embryonic stem cells; however, the localization of YTHDF2 genes in the stress granules was obviously reduced. Thus, the localization of YTHDF2 to stress granules under stress conditions relies on its modification with m^6A; that is, the subcellular localization of YTHDF2 gene is regulated by m^6A-modified mRNAs. The translation efficiency of m^6A-modified mRNA in methyltransferase14-knockout cells was obviously inhibited when cells are exposed to heat shock. Summarizing the above findings, Ries et al.\(^3\) affirmed that the RNA modification by m^6A, especially polymethylated m^6A-mRNAs, could recruit multiple YTHDF2 genes, and obviously enhanced the phase separation of YTHDF gene. Conversely, the phase separation of YTHDF2 was found to regulate the translation efficiency of polymethylated mRNA. Ultimately, these findings proposed a novel theory for the precision regulation of m^6A modification on cellular biological processes.

In addition, Fu et al.\(^16\) recently shown that when cells were under oxidative stress, a large number of m^6A-modified mRNAs were localized to stress granules. Additional studies indicated that YTHDF gene was very important for the formation of stress particles under oxidative stress. After knocking down of YTHDF by small interfering RNA, the level of stress granules decreased markedly. Interestingly, an article published in Nature proved that the knock-out of METTL14 would not affect the stress granules’ formation under heat shock conditions.\(^3\) While the article from bioRxiv indicated that the binding of YTHDF and m^6A was hindered,\(^16\) and
stress particle formation was markedly reduced. In other words, stress granules’ formation under thermal and oxidative stress may be triggered by dissimilar genes; the underlying precising regulatory mechanisms require further analysis.4 The two articles drew the reasonable conclusion from different perspectives; that is, m^6^A modification can promote the phase separation process of specific RNA-binding proteins.

Recently, Gao et al.17 found that multivalent m^6^A-containing RNA can increase the phase separation YTHDF genes in HEK293 cells. Their data indicated that the m^6^A promotion of phase separation of YTHDF proteins is related to the response of cells to stress. YTHDF1/2/3 comprise a family of m^6^A readers. The researchers first analyzed the sequence of these three proteins and found a large number of low complexity domains. Then, they verified that both the low complexity domains and full-length proteins of YTHDF exhibited phase separation. m^6^A has a key role in diverse biological processes by binding to the relevant YTH domain-containing genes, as well as binding to other translation initiation factors. Multivalent m^6^A-driven phase separation of YTHDF genes may contribute to the physiology of a cell in the stress response. These studies provide an experimental basis for the regulation of m^6^A phase separation and gene expression regulation and also provide an understanding for the regulation of key gene expression during cell fate determination and disease development.18–20 At present, the structures of membrane-less organelles of phase separation are very clear, and a subsequent major objective is to investigate the distinct properties of phase separation. Concentrated states of DNA, protein, and RNA are a primary aspect of intracellular compartments.21 It has become commonly accepted that large amounts of these condensates form via LLPS, resulting in liquid-like states of intracellular matter. Thus, intracellular liquid condensates can drive richer structural assembly than uniform droplets from multilayer structures, such as nucleoli and stress particles, to liquid crystal assemblies, such as spindle-shaped and actin condensates.21,22

Physical and chemical properties of phase separation will be an increasingly important subject for future challenges. Employing m^6^A-modified mRNAs governed by phase separation as a target to regulate various cellular and physiological processes, such as transcriptional control, stress exposure, signal transduction, etc. will also provide a new insight for m^6^A-regulated phase separation in different research fields. Research on the relationship between phase separation and disease is progressing rapidly. The current research is primarily focusing on the molecular pathogenesis of neurodegenerative diseases.23 Some gene point mutations, such as FUS protein, can increase the transition from a solid phase to a liquid solvent and may be the reason for the formation of insoluble protein accumulation which is commonly found in neurodegenerative disorders.24 Moreover, Nuclear transport has been shown to decrease nucleic acid binding protein concentration in the cytoplasm, dissolve the LLPS structure, and prevent neurotoxicity formation attributed to solid aggregation.25

**Hypothesis**

Recent studies have indicated that the modification of m^6^A is involved in the regulation process of mRNA metabolism, in particular in modulating mRNA stability, determining cell fate, affecting different cellular biological systems, and taking a responsible role in cell fate determination, immunity, and lipid metabolism. Scientists have tried to explain the regulation mechanisms of m^6^A to the translation efficiency and stability of mRNA based on three of the major m^6^A-binding proteins. Primary gene sequence analysis showed that the YTHDF genes consisted of an m^6^A-binding YTH location of about 15 kDa and a low complexity location of about 40 kDa. Thus, the authors speculated that the YTHDF genes may undergo LLPS based on this structural arrangement (Fig. 2).

**Conclusions**

Phase separation is increasingly recognized as key regulatory biological macromolecules in cytoplasm. It is time to expand the research focus to phase separation for numerous biological functions. Studying the m^6^A-mediated phase separation mechanism, especially finding or screening key proteins will provide a theoretical basis for verifying the effectiveness of target macromolecules.

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**Conflict of interest**

The authors have no conflicts of interest related to this publication.

**Author contributions**

Designing, reviewing and revising the article (YFL), drafting of the manuscript (LSY), reviewing and drawing (FY, WJJ), collecting materials and revising the manuscript (YZD, ZML, SZL), approved the final version submitted (all the authors).

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