

Ribosomal frameshifting and misreading of mRNA in COVID-19 vaccines produces “off-target” proteins and immune responses eliciting safety concerns: Comment on UK study by Mulroney *et al.*

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## Capsule

We comment on the study by Mulroney *et al.*(1) entitled: “*N1-methylpseudouridylation of mRNA causes +1 ribosomal frameshifting.*” The study found evidence in mice and humans for the misreading of the modRNA contained within the Pfizer COVID-19 vaccine to inadvertently produce “off-target” proteins capable of eliciting “off-target” immune responses. The authors propose that these novel proteins are the result of ribosomal frameshifting occasioned by the substitution of N1-methyl pseudouridine. The authors state that the “error prone” code is a safety concern with a “*huge potential to be harmful*” and that “*it is essential that these therapeutics are designed to be free from unintended side-effects.*”

The findings reveal a developmental and regulatory failure to ask fundamental questions that could affect the safety and effectiveness of these products. According to WHO guidelines for mRNA vaccines, (2) manufacturers should provide details of “*unexpected ORFs*”(Open Reading Frames). *The formation of these off-target proteins is not disclosed in the package insert for COMIRNATY.* (3):

The finding that unintended proteins may be produced as a result of vaccination is sufficient cause for regulators to conduct full risk assessments of past or future harms that may have ensued. Given that this study was conducted under the auspices of the United Kingdom Government, we must assume UK regulators, manufacturers, and international regulatory agencies, including FDA, were apprised of the data many months ago. We await their account of what steps they have taken to investigate why the formation of off-target proteins was not discovered sooner, what toxic effects they may have caused and what steps they are taken to prevent harm in the future and to inform the public of these findings.

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## 1. Background

A paper was published today by Mulroney *et al.*(1) in the journal Nature entitled: “*N1-methylpseudouridylation of mRNA causes +1 ribosomal frameshifting.*” The study was led by Professor Anne Willis of the UK’s Medical Research Council Toxicology Unit at the University of Cambridge and included collaborators from Oxford, Dublin, Liverpool, Kent, and Thailand; some of whom are part of the UK’s National Institute for Health and Care Research.

The paper was accompanied by a [press release](#)<sup>1</sup> summarizing the study:

*“Researchers have discovered that misreading of therapeutic mRNAs by the cell’s decoding machinery can cause an unintended immune response in the body. They have identified the sequence within the mRNA that causes*

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<sup>1</sup> [www.cam.ac.uk/research/news/researchers-redesign-future-mrna-therapeutics-to-prevent-potentially-harmful-immune-responses](http://www.cam.ac.uk/research/news/researchers-redesign-future-mrna-therapeutics-to-prevent-potentially-harmful-immune-responses)

this to occur and found a way to prevent ‘off-target’ immune responses to enable the safer design of future mRNA therapeutics.”

The COVID-19 mRNA vaccines made by Pfizer-BioNTech and Moderna are referred to in regulatory documents (4) as “nucleoside modified RNA” (modRNA) vaccines because the uridine residues have been replaced with N1-methylpseudouridine (1-methyl $\Psi$ ). This substitution allows the modRNA to evade normal defense mechanisms that detect foreign RNA allowing the vaccinal RNA to enter the cell, ultimately to produce spike protein. The discovery of this fact led to the award of the Nobel Prize in Physiology and Medicine in 2023 to Katalin Karikó and Drew Weissman.

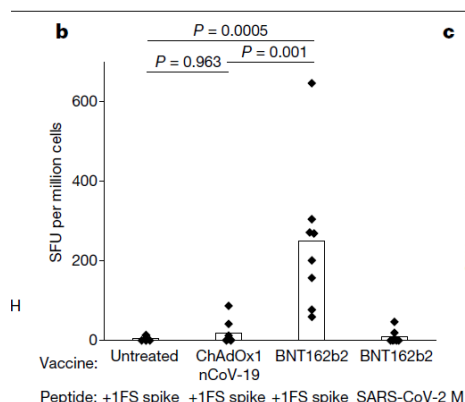
Mulroney *et al.*, noted in their introduction that pseudouridine ( $\Psi$ ) is known to increase misreading of mRNA. McKernan *et al.* also noted previously that “Pseudouridine is also known to create ribosomal frameshifts.” (5)

Mulroney *et al.*, noted further that “although 1-methyl $\Psi$  does not seem to affect codon misreading” it has been shown to “affect protein synthesis rates and ribosome density on mRNAs.”

The impetus for the study appears to be based on the authors’ observation that “So far, no study has investigated the fundamental question of whether modified ribonucleotides can affect the maintenance of the correct reading frame during translation of a synthetic transcript.”

## 2. Main Findings

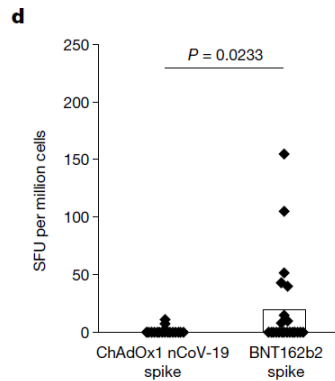
1. In an *in vitro* model system looking at the translation of a luciferase reporting gene in HeLa cells, RNA containing 1-methyl $\Psi$  significantly increased ribosomal +1 frameshifting to produce different proteins.
2. Mulroney *et al.*, vaccinated mice with the Pfizer COVID-19 vaccine (BNT162b2) and examined T-cell responses *in vitro* to proteins they synthesized based on the sequence produced by a +1 frameshift. Compared with the relevant controls, including cells challenged with spike protein and mice vaccinated with the Astra-Zeneca ChAdOx1 nCoV-19 vaccine, responses (which they termed “off-target immunity”) to +1 frameshifted spike peptides were significantly increased (Fig 2b there).



**Figure 2b from Mulroney *et al.* (1):** “Splenocyte IFN $\gamma$  ELISpot responses from untreated, ChAdOx1 nCoV-19-vaccinated or BNT162b2-vaccinated mice stimulated with +1FS spike peptides. IFN $\gamma$  ELISpot response from BNT162b2-vaccinated mice stimulated with SARS-CoV-2 M peptides (unrelated control antigen) is included for additional comparison. SFU, spot-forming units. Each group  $n = 8$ . Untreated versus ChAdOx1 nCoV-19,  $P = 0.963$ ; untreated versus BNT162b2,  $P = 0.0005$ ; ChAdOx1 nCoV-19 versus BNT162b2,  $P = 0.001$ .”

3. Mulroney *et al.*, repeated these findings from peripheral blood mononuclear cells obtained from 21 individuals vaccinated with BNT162b2 and 20 individuals vaccinated with ChAdOx1 nCoV-19, “none of whom reported undue effects as a result of vaccination.” (Figure 2d there) These subjects mainly were part of another study (SIREN)<sup>2</sup> funded by the UK Health Security Agency.

<sup>2</sup> <https://www.isrctn.com/ISRCTN11041050?q=SIREN&filters=&sort=&offset=2&totalResults=2&page=1&pageSize=10>  
<https://www.gov.uk/guidance/siren-study>



**Figure 2d from Mulrone et al. (1):** “Peripheral blood mononuclear cells (PBMC) IFN $\gamma$  ELISpot responses from donors vaccinated with ChAdOx1 nCoV-19 ( $n = 20$ ) or BNT162b2 ( $n = 21$ ) stimulated with +1FS spike peptides.  $P = 0.0233$  (Welch’s one-tailed  $t$ -test).”

These responses did not appear to be related to age, gender or HLA subtype.

Taken together, Mulrone concluded that “these data suggest that vaccination with 1-methyl. mRNA can elicit cellular immunity to peptide antigens produced by +1 ribosomal frameshifting in both major histocompatibility complex (MHC)-diverse people and MHC-uniform mice.”

4. *In vitro* translation experiments identified a number of frameshift peptides including a chimeric polypeptide consisting of in-frame N-terminal residues and +1 frameshifted C-terminal residues.
5. The authors identified several parts of the RNA sequence where frameshifting was likely to occur, and experimented with various modifications to reduce “slippage.” They noted that their data “suggest that N1-methylpseudouridylation at defined mRNA sequences triggers ribosome +1 frameshifting; however, with appropriate mRNA sequence design, it is possible to ameliorate this issue.”

### 3. Authors’ conclusions

Contained mainly in the paper’s conclusions and also in the press release are the following remarks:

- “When the ribosome is confronted with a string of these modified bases called N1-methylpseudouridine in the mRNA, it slips around 10% of the time causing the mRNA to be misread and unintended proteins to be produced – enough to trigger an immune response.”
- “Removing these runs of N1-methylpseudouridine from the mRNAs prevents ‘off-target’ protein production.”
- “Although there is no evidence that frameshifted products in humans generated from BNT162b2 vaccination are associated with adverse outcomes, for future use of mRNA technology it is important that mRNA sequence design is modified to reduce ribosome frameshifting events, as this may limit its future use for applications that require higher doses or more frequent dosing, such as the *in vivo* production of hormones.”
- “As billions of pounds flow into the next set of mRNA treatments, it is essential that these therapeutics are designed to be free from unintended side-effects.”
- “We can remove the error-prone code from the mRNA in vaccines so the body will make the proteins we want for an immune response without inadvertently making other proteins as well. The safety concern for future mRNA medicines is that mis-directed immunity has huge potential to be harmful, so off-target immune responses should always be avoided.”
- “Our work presents both a concern and a solution for this new type of medicine.”

#### 4. Commentary

The paper provides evidence for the formation “*off-target*” or unintended proteins following vaccination with BNT162b2 due to frameshifting. Given the proposed mechanism, a similar problem is likely to exist for the Moderna product.

While the authors have not isolated samples of these proteins from vaccinated patients or animals, their existence is evidenced by the specific cellular immune responses elicited to frameshifted proteins the authors synthesized. It is not clear why B cell – antibody responses were not studied.

The authors state that “*Although there is no evidence that frameshifted products in humans generated from BNT162b2 vaccination are associated with adverse outcomes.*” It is unclear how it is possible to make this statement, given:

- The small number of vaccinated subjects (n=21) providing samples.
- This was not a controlled trial.
- None of these subjects had reported undue effects of vaccination. Accordingly, the sample is subject to selection bias.
- The toxicology of these unintended proteins must be studied.
- The authors acknowledge the misdirected immunity “*has huge potential to be harmful.*”
- These proteins may already have contributed to vaccine toxicity, which now must be the subject of investigation.

The full sequence of these proteins should be provided. Further, the homologies between the proposed frameshifted proteins and peptides and known proteins must be conducted using databases and tools such as BLAST. One of the proteins identified was characterized as a chimeric protein. McKernan *et al.* (5) showed how in theory, a chimeric viral-human protein might be formed that has a homology similarity to a human protein called gp130, which forms part of a receptor for IL-6.

The premise for the study<sup>3</sup> reveals a developmental and regulatory failure to ask fundamental questions that could affect the safety and effectiveness of these products. This is no better exemplified by Pfizer’s retired head of vaccine R&D who was quoted in Nature as saying: “*We flew the aeroplane while we were still building it.*” (6)

According to WHO guidelines for mRNA vaccines, (2) manufacturers should provide details “*unexpected ORFs,*” (emphasis added).

“*The complete annotated sequence **identifying all ORFs (including any unexpected ORFs)** and all other sequence elements (including their justification for use) should be provided. Justifications for the use of any specific noncoding sequence and of structural elements such as the chosen 5` cap structure should be provided. [...] The anticipated function and purpose of each gene sequence encoded in the mRNA should be indicated, as well as those of specific noncoding and structural elements, explaining their contribution to the overall mode- or mechanism-of-action.*”

If Mulrone *et al.* were able to predict the existence of frameshifted proteins, why were Pfizer’s scientists unable to do so? The same question may be asked of regulators, especially in light of unresolved discrepancies and the specific obligation imposed by the European Medical Agency on BioNTech regarding the identities of the observed Western Blot (WB) bands obtained by *in vitro* expression assays.(7)

Documents disclosed under the FOIA (8) reveal that three categories of preclinical studies were not performed by Pfizer, relevant to the current findings: 1) secondary pharmacodynamics, 2) safety pharmacology and 3) pharmacodynamic drug interactions, In two of these categories, WHO guidelines were cited in justification (highlight added).

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<sup>3</sup> “*So far, no study has investigated the fundamental question of whether modified ribonucleotides can affect the maintenance of the correct reading frame during translation of a synthetic transcript.*”

#### 2.4.2.2. Secondary Pharmacodynamics

No secondary pharmacodynamics studies were conducted with BNT162b2.

#### 2.4.2.3. Safety Pharmacology

No safety pharmacology studies were conducted with BNT162b2 as they are not considered necessary for the development of vaccines according to the WHO guideline (WHO, 2005).

#### 2.4.2.4. Pharmacodynamic Drug Interactions

Nonclinical studies evaluating pharmacodynamic drug interactions with BNT162b2 were not conducted as they are generally not considered necessary to support development and licensure of vaccine products for infectious diseases (WHO, 2005).

The package insert for COMIRNATY states (3):

*“Each 0.3 mL dose of COMIRNATY (2023-2024 Formula) is formulated to contain 30 mcg of a nucleoside modified messenger RNA (modRNA) encoding the viral spike (S) glycoprotein of SARS-CoV-2 Omicron variant lineage XBB.1.5 (Omicron XBB.1.5).”*

There is no mention of any other kind of protein.

The finding that unintended proteins may be produced as a result of vaccination is sufficient cause for regulators to conduct full risk assessments of past or future harms that may have ensued. We note that regulators have previously failed to insist on the study and assessment of risk of the pharmacology and toxicology of novel spike protein heterotrimers forming after injection of the bivalent COVID-19 modRNA vaccines.(9)

The paper was received by Nature on January 25, 2023, accepted for publication on October 31, 2023 and published today, December 6, 2023. This time frame appears rather protracted given the significance of these findings. The study was funded and conducted by agencies of the United Kingdom. The evidence for the formation of off-target proteins must surely be considered a reportable adverse event.

We must assume UK regulators, manufacturers, and international regulatory agencies, including FDA, were apprised of the data many months ago. We await their account of what steps they have taken to investigate why the formation of off-target proteins was not discovered sooner, what toxic effects they may have caused and what steps they are taken to prevent harm in the future and to inform the public of these findings.

## 5. Revision history

V1 12/6/23

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