



Principles for designing an optimal mRNA lipid nanoparticle vaccine

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mRNA Lipid nanoparticles (LNPs) have recently been propelled onto the center stage of therapeutic platforms due to the success of the SARS-CoV-2 mRNA LNP vaccines (mRNA-1273 and BNT162b2), with billions of mRNA vaccine doses already shipped worldwide. While mRNA vaccines seem like an overnight success to some, they are in fact a result of decades of scientific research. The advantage of mRNA-LNP vaccines lies in the modularity of the platform and the rapid manufacturing capabilities. However, there is a multitude of choices to be made when designing an optimal mRNA-LNP vaccine regarding efficacy, stability and toxicity. Herein, we provide a brief on what we consider to be the most important aspects to cover when designing mRNA-LNPs from what is currently known and how to optimize them. Lastly, we give our perspective on which of these aspects is most crucial and what we believe are the next steps required to advance the field.

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Current Opinion in Biotechnology 2021, 77:xx–yy

This review comes from a themed issue on **Nanobiotechnology**

Edited by **Peter M Tessier** and **Ravi S Kane**

<https://doi.org/10.1016/j.copbio.2021.09.016>

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Introduction

Generally, mRNA-LNP design is comprised of several elements: (1) mRNA sequence design and nucleotide modification choice, (2) Optimization of a LNP formulation to encapsulate and deliver mRNA and (3) long-term

storage. Each one of these phases entails a plethora of choices and considerations to make. Currently, mRNA-LNPs are evaluated based on their ability to express encoded mRNA, their immunogenicity due to recognition of mRNA as a foreign entity by intracellular RNA sensors, LNP formulation stability and toxicity. All these are examined in light of the route of administration and therapeutic goal. Importantly, the design of an optimal therapeutic mRNA-LNP for systemic administration differs from an optimal intra-muscularly administered mRNA-LNP vaccine. We will focus on the optimal design of efficacious mRNA-LNP vaccines regarding expression, stability, and toxicity. We will summarize what is currently known from the literature, from our own experience and from comparison of the two recently greenlighted mRNA vaccines for SARS-CoV-2, Moderna's mRNA-1273 and Pfizer/BioNTech's BNT162b2. While many elaborative reviews covering the theories behind each component exist, we aim to provide a brief on what is currently known and what we deem to be most important.

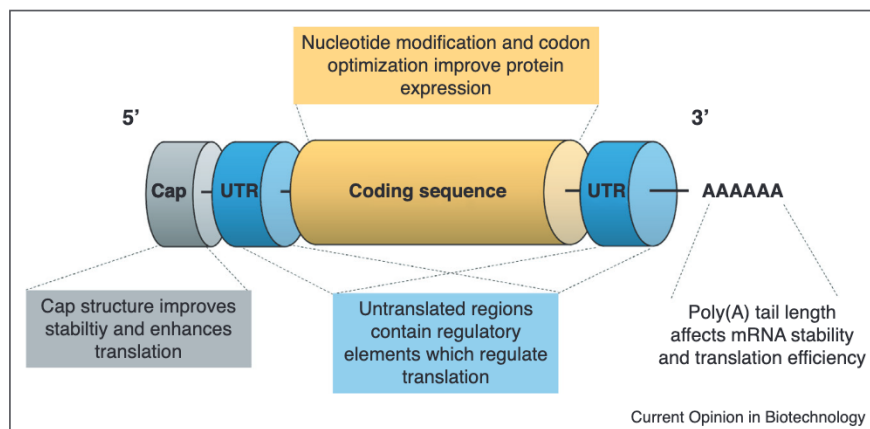
Optimizing mRNA sequence and modifications for mRNA-LNP vaccines

The main considerations for mRNA sequence and modification selection are: (1) expression of encoded mRNA, (2) immunogenicity due to recognition of mRNA as a foreign entity by intracellular RNA sensors and (3) mRNA stability. These are controlled by designing mRNA with modified nucleotides, sequence modifications and mRNA capping modalities (**Figure 1**).

Nucleotide modifications

Nucleotide modifications are considered the most important breakthrough that initiated the mRNA therapeutics field. Non-modified mRNA molecules are recognized by cellular RNA sensors which result in innate immunity activation [1,2]. While activation of innate immune pathways can be advantageous in the context of vaccination by serving as an adjuvant, these innate immune responses may also be detrimental to mRNA therapy, since they dramatically reduce mRNA translation [3,4]. In 2005, a groundbreaking study by Kariko and colleagues demonstrated that incorporation of naturally occurring, chemically modified nucleosides such as pseudouridine (Ψ), thiouridine (s2U), and 5-methylcytidine (m5C) resulted in a significant reduction in the immunogenicity of the mRNA [5]. Most importantly, several studies demonstrated that incorporation of modified nucleosides resulted in enhanced stability of the RNA molecule,

Figure 1



Optimizing mRNA sequence and modifications for mRNA-LNP vaccines.
A scheme representing the mRNA construct elements to consider when designing an mRNA-LNP vaccine.

and increased protein translation [6–8,9*], including the N1-methylpseudouridine modification which has been employed in both mRNA-1273 and BNT162b2 [10,11]. Contrastingly, CureVac's sequence-optimized candidate, CVnCoV, is exclusively composed of unmodified nucleosides. This approach implements sequence-engineered, unmodified mRNA, to provide a robust and balanced immune response. However, preliminary phase 2b/3 data indicates that the vaccine candidate was substantially less effective than the other two leading mRNA vaccines by Pfizer/BioNTech and Moderna) (NCT04652102) [12,13]. While the inferior efficacy of CureVac's vaccine candidate could be attributed to the inclusion of unmodified nucleosides, several other dissimilarities between the candidates should be taken into account, such as differences in non-coding elements and storage conditions (see Table 1). Therefore, when considering nucleotide modifications, there is a tradeoff to consider between potential innate adjuvant responses driven by unmodified nucleosides and enhanced protein expression attributed to modified nucleosides. Currently, both FDA authorized mRNA vaccines incorporate modified nucleotides.

mRNA capping

mRNA capping dramatically increases translation efficiency and intra-cellular mRNA stability by binding to the eukaryotic translation initiation factor 4E (eIF4E) [14,15]. Capping of *in vitro* transcribed (IVT) mRNA is frequently performed using a cap analog, which can be added during the IVT process or post-transcription. However, the mRNA can be reversely capped which results in rapid degradation and poor translation. To avoid reverse 5' cap incorporation, anti-reverse cap analogs (ARCA) have been developed, which ensure correct capping orientation [16]. Further modifications were developed over the years to improve the performance of ARCA, with

the recently developed 'CleanCap' analog (TriLink Biotechnologies, San Diego, CA, USA) most frequently used nowadays in mRNA companies [17]. Therefore, stable, correctly oriented caps need to be incorporated for an effective mRNA-LNP vaccine.

UTR selection

UTR selection also needs to be considered, since they greatly affect mRNA decay and translational efficiency. 5' UTR features such as start codons and secondary structures may compromise ribosome recruitment, scanning and start codon recognition, and thus should be avoided. Grossly, the 5' UTR sequences are critical for protein expression while the 3' UTRs are more likely to affect mRNA half-life [18–20]. For example, the β globin 3' UTR and a duplication of the β -globin 3' UTR stabilize mRNA [21].

ORF design

ORF sequence design also carries a critical impact on translation efficiency and immunogenicity of the mRNA due to recognition by cellular sensors. In addition to the nucleotide modifications mentioned, codon optimization has been demonstrated to significantly enhance protein expression by incorporating frequent codons and/or codons with higher tRNA abundances [22]. Another form of sequence optimization is enrichment of guanine-cytosine (GC) content, which has been shown to increase steady-state mRNA levels *in vitro* and protein expression *in vivo*, and has been employed by CureVac in its recent SARS-CoV-2 mRNA vaccine candidate, CVnCoV (NCT04652102) [22,23]. Furthermore, the mRNA sequence dictates the secondary structure. Interestingly, this secondary structure can influence mRNA degradation by hydrolysis. Recently, specially designed algorithms that design optimal mRNA sequences for maximal

Table 1
Comparison of mRNA and LNP formulation elements of three SARS-CoV-2 mRNA LNP vaccines

	BNT162b2 (Pfizer/BioNTech)	mRNA-1273 (Moderna)	CVnCoV (CureVac)
Approval Status [48–50]	FDA Approved August 23, 2021	Emergency Use Authorization (EUA) December 18, 2020 Submitted for full Approval August 25, 2021	Phase 2b/3 (NCT04652102)
Lipid Formulation (% Molar ratio of ionizable: Cholesterol: Neutral lipid: PEGylated lipid) [9*]	46.3:42.7:9.4:1.6	50:38.5:10:1.5	50:38.5:10:1.5
N:P Molar Ratio [9*]	6		
Cap [51–53]		Cap-1	
5'-UTR [54]	5' -untranslated region derived from human alpha-globin RNA with an optimized Kozak sequence	Undisclosed	5' UTR: Artifacts from restriction and transcription site, plus Kozak sequence
S protein antigen [53,55]	Codon-optimized sequence encoding full-length SARS-CoV-2 spike (S) glycoprotein containing mutations K986P and V987P	Codon-optimized sequence encoding full-length SARS-CoV-2 spike (S) glycoprotein containing mutations K986P and V987P	Codon-optimized and engineered sequence encoding full-length SARS-CoV-2 spike (S) glycoprotein containing mutations K986P and V987P
Modified nucleotides [54]	N1-methyl-pseudouridine	N1-methyl-pseudouridine	Unmodified nucleotides
3'-UTR [54]	3' UTR comprising two sequence elements derived from the amino-terminal enhancer of split (AES) mRNA and the mitochondrial encoded 12S ribosomal RNA	Undisclosed	3' UTR comprising human alpha-globin 3' UTR sequence element
Poly(A) Tail [52,53]	A 110-nucleotide poly(A)-tail consisting of a stretch of 30 adenosine residues, followed by a 10-nucleotide linker sequence and another 70 adenosine residues.	Undisclosed	64 Poly (A) tail
Storage [9*,56]	–60°C to –80°C for six months –15°C to –25°C for two weeks 2°C–8°C for 5 days	–15°C to –20°C for six months 2°C–8°C for 30 days	2°C–8°C for three months
Buffer [9*]	PBS	Tris	Undisclosed
Cryoprotectant [9*]	Sucrose	Sucrose	Undisclosed

base stacking regions have been reported to significantly improve mRNA stability [24*].

Poly(A) tail

The poly(A) tail also contributes to mRNA translation and stability by reducing RNA exonuclease activity. In addition, the poly(A) tail binds to poly(A)-binding proteins (PABP), which recruit eIF4G and eIF4E, increasing the affinity to the mRNA cap and promoting a circular mRNA structure and efficient translation. Contrastingly, PABP has also been shown to participate in microRNA-mediated inhibition of translation [25]. When considering the length of the poly(A) tails, there is no consensus. While it is suggested that longer poly(A) tails (120–150 nucleotides) increase mRNA stability, the dual effect of PABP-mediated inhibition of translation warrants further optimization. For example, Lima *et al.* demonstrated that mRNAs with high translation efficiency actually had short poly(A) sequences (~33–34 nucleotides) [25]. mRNA created by IVT can be polyadenylated in two ways: either by encoding the poly(A) on the DNA template used or by enzymatic addition by poly(A) polymerase to the mRNA

after IVT. Encoding the poly(A) tail on the DNA plasmid ensures the production of a defined poly(A) tail length, whereas enzymatic polyadenylation of the mRNA produces varying lengths of poly(A) tails and is therefore, less favorable.

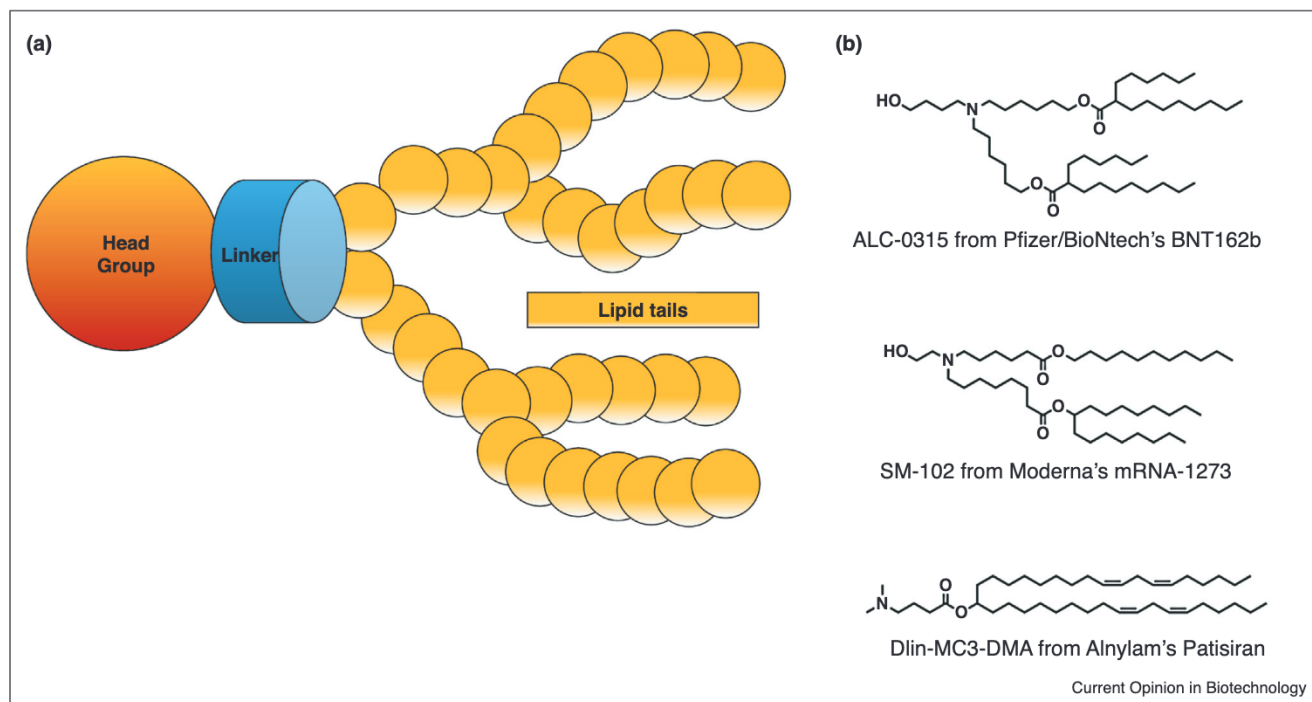
LNP formulation considerations

LNPs, the mRNA vehicles, both protect mRNA payloads from degradation and enable their efficient delivery into target cells. Generally, LNPs are a lipid formulation comprised of various ratios of a neutral structural lipid, cholesterol, a PEG-lipid and the ionizable lipid.

Ionizable lipid

The ionizable lipid is considered the most important component and generally sets apart different mRNA-LNPs. Libraries of ionizable lipids are constantly screened to find the optimal lipid to enhance expression and provide better immune responses in mRNA-LNP vaccines while retaining low toxicity profiles [26,27]. Ionizable lipids are comprised of: (1) an ionizable head group, (2) a linker region and (3) the hydrocarbon chains

Figure 2



LNP formulation considerations.

(a) A scheme representing the elements of the ionizable lipid. (b) Chemical structures of the three clinically approved ionizable lipids. ALC-0315 (Pfizer/BioNtech's BNT162b), SM-102 (Moderna's SM-102) and Dlin-MC3-DMA (siRNA LNPs — Alnylam's Patisiran) [34].

(Figure 2a). While ionizable lipids structures vary, some common grounds for effective ionizable lipids for mRNA-LNP vaccines have emerged.

The ionizable head group: mRNA-1273 and BNT162b2 vaccines each comprise a different ionizable lipid, however, they both contain an amino-alcohol head group with pKas of 6.75 and 6.09, respectively [28]. A study by Moderna claimed that the optimal range of ionizable lipid pKa for eliciting an adaptive immune response following an mRNA vaccine is 6.6–6.9, whereas a pKa range of 6.2–6.6 has been shown to be optimal for protein expression following IV delivery [27]. Interestingly, this is consistent with mRNA-1273 ionizable lipid's pKa, yet not with BNT162b2.

The linker region connects the head group with the lipid hydrocarbon tails, also gains attention for its effect on the *in-vivo* activity of RNA-LNPs. It is currently hypothesized that the linker region contributes to head group pKa and RNA-LNP endosomal escape potential [29]. Research into linker region optimization is an ongoing process. Ionizable lipid libraries with various linker structures have been designed and screened for their ability to delivery of RNA-LNPs more efficiently [30].

The lipid tails of the ionizable lipids are also to be considered. Lipid structure (and hence LNP structure) affects expression efficiency, by altering endosomal escape, stability during storage and toxicity [31]. Interestingly, studies have claimed correlations between various unsaturation degrees and symmetry of hydrophobic tails to more efficient and stable LNPs. For example, branched hydrocarbon lipid tails may create a more cone-shaped structure that enhances endosomal escape [32*]. Furthermore, ester linkages are introduced into lipid hydrocarbon chains to improve the ionizable lipid's pharmacokinetic properties. The first FDA-approved ionizable lipid Dlin-MC3-DMA (MC3) raised some concerns due to its long tissue half-life post-administration. This propelled the development of biodegradable ionizable lipids to improve lipid metabolism and prevent toxicities. Introducing one or more ester linkages, in the hydrocarbon tails as well as the linker region, leads to *in-vivo* cleavage by esterases, which dramatically improves the pharmacokinetic properties of the lipids, and reduces toxicities [26]. Studies constantly attempt to find the optimal ester bond position in the chains. It is assumed that placing these bonds too close to the head group-linker region reduces overall LNP efficacy by altering head group pKa. Therefore, it is preferable to locate them further downstream in the lipid tails [26,29]. Currently, both mRNA-1273 and

BNT162b2 contain branched hydrocarbon tails with biodegradable linkages (Figure 2b).

Polyethylene glycol (PEG)-lipid

Polyethylene glycol (PEG) lipids generally comprise <2.5% of the total formulation. Nevertheless, they play a key role in controlling particle size and elongate shelf-life stability by preventing aggregation and fusion of LNPs. However, PEG-lipids need to be balanced since at high concentrations they prevent the delivery of RNA into cells. Recently, there have been some reports of immune responses to PEG molecules which have prompted the search for safe and potent PEG alternatives for mRNA LNPs, such as polysarcosine [33**]. Both mRNA-1273 and BNT162b2 contain 1.5 and 1.6% PEG-lipid in formulation, respectively [9].

Long-term preservation

The long-term storage and stability of mRNA-LNPs is a major consideration in the design of the formulations, and one which hasn't been explored thoroughly, given its high clinical relevance and effect on vaccine distribution and ultimately, price. It's astonishing to look at the speed of development of Moderna's mRNA-1273, the first mRNA vaccine for SARS-CoV-2 to reach clinical trials. From the first viral genome publication, it took a mere two days to finalize the vaccine sequence, 25 days to manufacture the first dose and 63 days until the first participant was dosed in the Phase 1 clinical study (NCT04283461) [35]. However, scaling up to supply a global vaccine demand is challenging [36]. The ultra-low storage requirement of currently approved mRNA vaccines (-80°C for BNT162b2 and -20°C for mRNA-1273) due to mRNA-LNP instability at room temperature is a system constraint. Several studies have been carried out with the purpose of finding optimal LNP freezing conditions. Currently, it is accepted that to freeze LNPs, cryoprotectants such as sucrose or trehalose should be added after mRNA-LNP formulation [37]. Zhao *et al.* investigated the long-term storage conditions of lipid-like nanoparticles (LLNs). They report that the addition of 5% sucrose or trehalose to LLNs-mRNA formulations stored in liquid nitrogen, preserved the physicochemical properties of the particles, and allowed maintenance of *in vivo* delivery efficacy for three months [38].

Lyophilization of mRNA LNPs can also be accomplished by the addition of lyoprotectants. For example, lyophilization provides Moderna's phase II mRNA-LNP CMV vaccine (mRNA-1647) stability for at least 18 months at 5°C , however, lyophilization is an expensive and time-consuming process [39,40].

Currently, both mRNA-1273 and BNT162b contain 10% final concentration of sucrose in the final product, pre-dilution [9*]. Of note, although sucrose is currently used as a cryoprotectant in both authorized COVID-19 mRNA vaccines, initial temperature requirements were different, with Moderna's mRNA-1273 stored at -15°C to -20°C and Pfizer/BioNTech's BNT162b2 at -60°C to -80°C . Recent stability data has prompted an approval by the European Medicines Agency (EMA) to store the BNT162b2 vaccine at -15°C to -25°C for two weeks [34]. While distribution and storage requirements have been solved from an engineering perspective by designing temperature-controlled shipping containers, biotechnological advances to improve stability at non-frozen temperatures are still in demand.

A current hypothesis claims that freezing the LNPs protects the mRNA strands within the LNPs rather than stabilizes the LNPs themselves. LNPs may degrade over time by aggregation, fusion or leakage of mRNA from the LNPs, however, aggregation and fusion can be solved by the addition of stabilizing lipids such as PEG and mRNA leakage hasn't been previously reported [9*]. Therefore, while LNP stability is important, it is not the limiting factor for short-term non-frozen LNP stability. mRNA degradation within LNPs occurs at a faster rate and dictates the storage time and temperature. This occurs mainly via chemical degradation by hydrolysis of the nucleic acid phosphodiester backbone and oxidation in presence of water or acids/bases. Initially, LNPs encapsulating smaller RNA payloads such as siRNA contained a single phospholipid outer layer with ionizable lipids inverted internally with little to no water entrapped. However, recent reports suggest that LNP-encapsulated mRNA contain within their core ionizable lipids, cholesterol and water. Some studies indicate that up to 24% of the LNP core is made of water, which affects mRNA stability within the LNP [41]. This can perhaps explain why FDA-approved siRNA LNPs (Patisiran) can be stored at $2-8^{\circ}\text{C}$ for 24 months [42] while current mRNA-LNP COVID vaccines can be stored for one month at this temperature pre-dilution [43,44].

Conclusion and General guidelines

The successful design of effective mRNA-LNPs encompasses multiple considerations and choices which need to be made in accordance with the therapeutic purpose. While there is no single recipe for a successful mRNA-LNP, some common grounds do emerge when analyzing what is currently known on successful mRNA vaccines (Table 1).

In terms of mRNA sequence, modified and mRNA optimized sequences are required for maximal protein expression. Regarding formulation, currently approved mRNA LNP vaccines contain similar ratios of lipids with

a varying ionizable lipid and PEG-Lipid. Even though the ionizable lipid is not identical in both vaccines, they are somewhat structurally similar regarding the amino-alcohol head group and branched hydrocarbon lipid tails with ester bonds.

Interestingly, while studies are constantly attempting to enhance protein expression from mRNA-LNPs [45], it seems that for vaccine purposes there is a weak correlation between the formulation's protein expression level and the ability to elicit adaptive immune responses [27,46*]. Therefore, the endpoint for screening formulations should be the ability to elicit an adaptive immune response and not the overall protein expression.

Stability and long-term storage are crucial for mRNA-LNP formulations. From an analysis of the global health innovation center at duke university, an estimated 4.1 Billion mRNA-LNP vaccine doses are planned to be manufactured during 2021, note that the entire SARS-CoV-2 vaccine landscape is projected at 12 Billion doses for this year [47**]. The requirement to store these vaccines at ultra-low temperatures is challenging. A current hypothesis claims that the critical point is the mRNA strand stability within the LNP rather than the vehicle stability. Therefore, while algorithmic optimization of mRNA structure and modifications can improve stability, the best current vaccines should be formulated with cryoprotectants such as sucrose and novel solutions for LNP stability at non-frozen temperatures are still in demand.

To sum up, mRNA-LNP vaccines are an effective modular vaccine platform that enables rapid manufacturing of new vaccines and should be optimally designed considering the therapeutic target.

Conflict of interest statement

The authors declare the following competing financial interest(s): D.P. receives licensing fees (to patents on which he is an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) for, lectured (and received a fee) or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNtech RNA Pharmaceuticals, Centricus, Diagnostear Ltd., EPM Inc., Earli Inc., Impetis Biosciences, Kernal Biologics, Newphase Ltd., NLC Pharma Ltd., NanoGhosts Ltd., Roche, SirTLabs Corporation, and Teva Pharmaceuticals Inc.

CRedit authorship contribution statement

Edo Kon: Conceptualization, Writing - original draft. **Uri Elia:** Conceptualization, Writing - original draft. **Dan Peer:** Conceptualization, Writing - original draft.

Acknowledgement

EK thanks the Yoran Institute for Human Genome Research for their support.

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