RESEARCH ARTICLE

DETERMINATION OF THE TRANSBILAYER DISTRIBUTION OF PLASMA MEMBRANE CHOLESTEROL

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Synopsis

We propose that the distribution of cholesterol across the plasma membrane bilayer is determined by the sterol-phospholipid complexes in the two leaflets that are filled homeostatically to stoichiometric equivalence. Leaflet cholesterol is then the product of the abundance of its phospholipids and its sterol stoichiometry. The model predicts that two thirds of the cholesterol in the human erythrocyte membrane bilayer is located in the outer leaflet. The model also correctly predicts the cholesterol content of the bilayer as a whole.

ABSTRACT

The transbilayer distribution of plasma membrane cholesterol remains uncertain despite repeated analysis. Here we propose a new mechanism driving cholesterol sidedness: sterols form simple stoichiometric complexes with phospholipids. The model postulates that the phospholipids in the plasma membrane bilayer are fully complexed with cholesterol. The sterol in each leaflet is then the product of the abundance of its phospholipid and its sterol stoichiometry. Notably, lipid affinities are not relevant. Using literature values for the composition, abundance and sterol stoichiometry of the phospholipid in each leaflet, the model predicts that two thirds of the cholesterol in the human erythrocyte membrane bilayer is located in its outer leaflet, an exofacial to endofacial ratio of 2:1. The model also predicts that the cholesterol content of the bilayer as a whole is 0.75 mole/mole phospholipid; this matches literature values. The model suggests, furthermore, that the areas of its two leaflets are about the same. The concordance of prediction with observation validates the model and the values used for the parameters. The sterol in the exofacial leaflet of the plasma membrane of any cell is predicted to exceed that on its contralateral side when its phospholipids have a higher sterol stoichiometry and are fully complexed.

1 | Introduction

The transbilayer distribution of the major lipids in the plasma membrane has been studied extensively, particularly in the human erythrocyte [1-8]. The phospholipids are asymmetrically disposed with almost all the sphingomyelin and phosphatidylcholine located in the outer leaflet and the phosphatidylserine, phosphatidylethanolamine and phosphatidylinositides in the cytoplasmic leaflet [1, 2, 9-11]. However, the transbilayer distribution of the sterol remains unresolved. Various studies have suggested that cholesterol is located mostly in one leaflet or the other or in a roughly even distribution [3, 6, 12, 13]. Consensus is similarly lacking regarding

the mechanism driving the sidedness of plasma membrane cholesterol. Some have proposed that the outer leaflet has most of the cholesterol because it is enriched in saturated phospholipids with a relatively high sterol affinity [3, 8, 12, 14, 15]. Others have suggested that the sterol is pumped from the inner to the outer leaflet [16, 17]. Another contention is that a relative paucity of exofacial phospholipids is compensated for by extra cholesterol so as to equalize the surface area of the two leaflets and relieve interfacial stress [7, 8]. Alternatively, long chain sphingomyelins in the exofacial leaflet might drive cholesterol to the contralateral side [18]. Theoretical modeling and simulations have implicated the material properties of the bilayer in determining cholesterol sidedness [8, 12-14, 19, 20].

We now propose a new mechanism governing the transbilayer distribution of plasma membrane cholesterol. It is based on the premise that sterols and phospholipids form stoichiometric complexes [15, 21-23]. As noted above, the transverse asymmetry of the phospholipids has been well characterized, at least in the red cell. We reason that, if the cholesterol in each leaflet is fully complexed with the phospholipids at their stoichiometric equivalence point, the distribution of the phospholipids will determine that of the sterol. Based on this assumption, a data-driven model is proposed. It predicts that two thirds of the cholesterol in the human erythrocyte bilayer is exofacial. The stoichiometric complex model differs from some theoretical and computational approaches in doing without the sterol affinity of the phospholipids and membrane stress. It also offers two results that other treatments lack: it correctly predicts the cholesterol concentration in the membrane and satisfies the expectation that the areas of the two leaflets are about the same.

Stoichiometric complexes between sterols and phospholipids have not been definitively demonstrated. However, as detailed in the Discussion, their association has the requisite characteristics. That is, the interaction of sterols and phospholipids has a demonstrable affinity

and an apparent stoichiometry. In addition, it is subject to competition by other ligands. Furthermore, isotherms for the binding of cholesterol to phospholipids invariably have a threshold at an apparent stoichiometric equivalence point beyond which the excess sterol has a steadily increasing chemical activity and availability to probes. Lacking an alternative mechanism for the associations of phospholipids and cholesterol, it is described for now as stoichiometric complexes.

2 | Method

The stoichiometric complex model makes the following assumptions. (1) Cholesterol is in diffusional equilibrium within and between the liquid leaflets of the plasma membrane bilayer [23, 24]. (2) Cholesterol forms simple stoichiometric complexes with bilayer phospholipids [15, 21, 23, 25]. (3) Plasma membrane cholesterol is maintained homeostatically at the stoichiometric equivalence point of the phospholipids [23, 24, 26]. (4) It follows that the cholesterol/phospholipid concentration in each leaflet is equal to the stoichiometry of its complexes. Consequently, the abundance of the cholesterol in each leaflet is the product of the abundance of its phospholipid and the stoichiometry of its complexes. (Only when the phospholipids are incompletely filled with cholesterol do their affinities affect their fractional saturation and hence the distribution of the sterol among them.) Glycosphingolipids and low abundance phospholipids are ignored for want of sufficient data.

We define C_t, P_t, C_o, P_o, C_i and P_i as the total (t) moles of cholesterol and phospholipid in the bilayer and in its outer (o) and inner (i) leaflets, respectively. The phospholipids in each compartment are fully complexed with cholesterol at stoichiometries n_o and n_i mole/mole. We express this requirement as C/P= C:P. It follows that the cholesterol in the leaflets can be expressed as

$$C_o = n_o P_o \qquad (1)$$

$$C_i = n_i P_i \quad (2)$$

The overall bilayer cholesterol to phospholipid ratio, α is given by:

$$\alpha = C_t/P_t = C_t{:}P_t = (C_o + C_i)/(P_o + P_i) \quad (3)$$

Then, substituting from Eqs. 1 and 2,

$$\alpha = (n_o P_o + n_i P_i)/(P_o + P_i) \quad (4)$$

Rearranging gives

$$n_o = [\alpha(P_o + P_i) - n_i P_i]/P_o \quad (5)$$

$$n_i = [\alpha(P_o + P_i) - n_o P_o]/P_i \quad (6)$$

$$C_o = n_o P_o = \alpha(P_o + P_i) - n_i P_i \quad (7)$$

$$C_i = n_i P_i = \alpha(P_o + P_i) - n_o P_o \quad (8)$$

Table 1 outlines how the stoichiometric complex model predicts the concentration and transbilayer distribution of sterols in plasma membranes; the subsequent tables apply the model. Rows 1 and 2 enter assumed values for the amount and stoichiometry of the phospholipid in each leaflet. The crux of the model is that because the phospholipids are filled to capacity, the concentration of cholesterol (C/P) in each of the bilayer leaflets is the same as the stoichiometry of its phospholipids (C:P). It follows that the cholesterol content in each leaflet in row 3 is given by the corresponding phospholipid abundance in row 1 multiplied by its stoichiometry in row 2. Row 4 gives the cholesterol concentrations, C/P, by dividing row 3 by row 1. The values for the bilayer as a whole (namely, C_t and C_t/P_t) are calculated from the values for the two leaflets.

The model invokes neither membrane stress nor material properties such as intrinsic and spontaneous curvature, inter-leaflet coupling or a difference in leaflet area [8, 12-14, 19, 20]. On the other hand, imposition of any of these factors could alter the bilayer sterol asymmetry driven by the stoichiometric complexes.

Table 1 | The stoichiometric complex model

ROW	PARAMETER	BILAYER	OUTER LEAFLET	INNER LEAFLET
1	Phospholipid, moles P	P_t	P_o	P_i
2	C:P stoichiometry, mole/mole	n_t	n_o	n_i
3	Cholesterol, moles C	n_tP_t	n_oP_o	n_iP_i
4	C/P concentration, mole/mole	C_t/P_t	C_o/P_o	C_i/P_i

3 | Results

We applied the model to the human erythrocyte membrane in Table 2. The abundance of lipids is expressed in moles and the total bilayer phospholipid, P_t, is set at 1.0 mole. Concentrations and stoichiometries are in mole/mole. We estimated the distribution of phospholipids between the two leaflets in row 1 by assuming that the outer leaflet is composed entirely of sphingomyelin and phosphatidylcholine [1, 5]. These two kinds of phospholipids contribute slightly more than half of the total [4, 27]. We therefore set the abundance of the phospholipid in the outer leaflet at P_o = 0.50 moles. The inner leaflet would then contain the other phospholipid species plus a few percent each of the phosphatidylcholine and sphingomyelin [1]; they add up to P_i = 0.50 moles.

The outer leaflet C_o:P_o in row 2 of Table 2 was assigned the stoichiometry of its major constituents; namely, phosphatidylcholine and sphingomyelin, 1.0 mole/mole [25]. The stoichiometry of the complexes in the cytoplasmic leaflet was assigned the value experimentally determined for dioleoylphosphatidylcholine, C_i:P_i = 0.5 mole/mole, taken to represent the unsaturated phospholipids predominant in this leaflet [4, 25]. Since $P_o = P_i$, the overall

stoichiometry of the bilayer is simply the average of C_o:P_o and C_i:P_i ; that is, C_t:P_t = 0.75 (row 2 in Table 2).

The abundance of the cholesterol in each leaflet (row 3) was obtained by multiplying the abundance of the phospholipid in each compartment (row 1) by its corresponding sterol stoichiometry in row 2 (Eqs. 1 and 2). This gives values for the cholesterol in the outer and inner leaflets of C_o = 0.50 mole x 1.0 mole/mole = 0.50 moles and C_i = 0.50 mole x 0.50 mole/mole = 0.25 moles. These are the values given by Eqs. 7 and 8 as well. They are also the differences between the total and the leaflet cholesterol values, as they must be. Row 4 gives the cholesterol concentration (C/P) obtained by dividing the values in row 3 by those in row 1. Concentrations can be converted to mole fractions, C/(C+P).

Table 2 | The stoichiometric complex model applied to human erythrocyte bilayers

ROW	PARAMETER	BILAYER	OUTER LEAFLET	INNER LEAFLET
1	Phospholipid, moles P	1.00	0.50	0.50
2	C:P stoichiometry, mole/mole	0.75	1.00	0.50
3	Cholesterol, moles C	0.75	0.50	0.25
4	C/P concentration, mole/mole	0.75	1.00	0.50

Row 3 in Table 2 delivers the results of interest. The outer and inner leaflets of the human erythrocyte bilayer are predicted to contain 67% and 33% of the cholesterol respectively; hence, C_o/C_i = 2. The concentration predicted for the total bilayer cholesterol is C_t/P_t = 0.75. This value matches the experimental results for red cells: $C_t/P_t \approx$ 0.70-0.80 [7, 27-30].

We also tested the plausibility of the model by estimating the relative surface areas of the two leaflets of the bilayer predicted by the values in Table 2. If the composition of the outer leaflet is

taken as 0.50 moles of dipalmitoylphosphatidylcholine plus 0.50 moles of cholesterol, it will have a molecular cross-section of ~39 $Å^2$/molecule at a surface pressure of 30 mN/m, a typical value for plasma membranes [12, 31]. The relative area of the outer leaflet is therefore approximately (0.50 + 0.50) x 39 $Å^2$ = 39 $Å^2$. The endofacial leaflet is less saturated and less condensed than the outer leaflet [4]. If, from Table 2, it is composed of 0.50 moles of dioleoylphosphatidylcholine plus 0.25 moles of cholesterol, it will have a molecular cross-section of ~50 $Å^2$/molecule at a surface pressure of 30 mN/m [12, 32]. The relative area of the inner leaflet is therefore approximately (0.50 + 0.25) x 50 $Å^2$ = 37.5 $Å^2$. Thus, despite the uncertainties, idealizations and approximations of the estimates, our results meet the expectation that the two bilayer leaflets have closely similar areas.

The tidy outcome in Table 2 may not be accurate or apply to other cells. We therefore tested a few alternative values. In Table 3, we changed the stoichiometry of the outer leaflet in row 2 to 0.90 mole/mole and kept the other parameters the same. The resulting bilayer cholesterol concentration was C_t/P_t = 0.70. This is within the physiological range of $C_t/P_t \approx$ 0.70-0.80 [7, 27-30]. In this scenario, 64% of the cholesterol was exofacial and the cholesterol asymmetry was C_o/C_i = 1.8 instead of 2.0. Thus, the model tolerates modest variation in the stoichiometry of the complexes.

Table 3 | The stoichiometric complex model for bilayers with a different stoichiometry

Row	PARAMETER	BILAYER	OUTER	INNER
1	Phospholipid, moles P	1.00	0.50	0.50
2	C:P stoichiometry, mole/mole	0.70	0.90	0.50
3	Cholesterol, moles C	0.70	0.45	0.25
4	C/P concentration, mole/mole	0.70	0.90	0.50

The abundance of the phospholipids in the two leaflets of the plasma membrane might not always be equal. The cholesterol concentration in the bilayer, C_t/P_t, is then given by the general expression derived from Eq. (3),

$$C_t/P_t = P_oP_i\,(n_i/P_o + n_o/P_i)/(P_o + P_i) \qquad (9)$$

An illustration is provided in Table 4. Here, the values of P_o and P_i in row 1 have been changed to 0.55 and 0.45 moles. Because $P_o \neq P_i$, the value for C_t/P_t in row 2 is not the average of C_o/P_o and C_i/P_i. As given by Eq. (9), it has the value C_t/P_t = 0.775 mole/mole. This prediction is well within the experimentally observed range of $C_t/P_t \approx$ 0.70-0.80 [7, 27-30]. The ratio of outer to inner leaflet cholesterol in this case is 2.4 with 71% of the cholesterol in the outer leaflet. Thus, the model yields results compatible with the experimentally-determined erythrocyte membrane cholesterol concentration at least over a small range of values for the stoichiometry and transbilayer distribution of the phospholipids.

Table 4 | The stoichiometric complex model for bilayers with unequal P_o and P_i

Row	PARAMETER	BILAYER	OUTER	INNER
1	Phospholipid, moles P	1.00	0.55	0.45
2	C:P stoichiometry, mole/mole	0.775	1.00	0.50
3	Cholesterol, moles C	0.775	0.55	0.225
4	C/P concentration, mole/mole	0.775	1.00	0.50

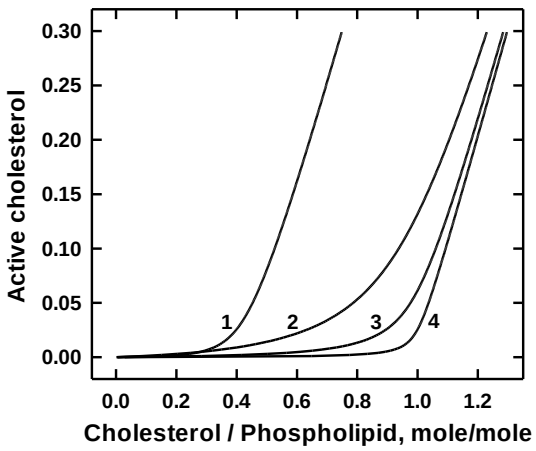
4 | Discussion

We have estimated the transverse asymmetry of cholesterol in the human red cell membrane bilayer by applying experimental data from the literature to a mechanistic model based on the premise that the phospholipids are fully complexed with cholesterol. The result was that two thirds of the bilayer cholesterol resides in the outer leaflet of the bilayer (Table 2), reflecting its

enrichment in saturated phospholipids with a high sterol complexation stoichiometry. The predicted total bilayer cholesterol concentration, $C_t/P_t = 0.75$, matches the observed value, $C_t/P_t \approx 0.70\text{-}0.80$ [7, 27-30], substantiating the premise that the bilayer sterol rests at its overall stoichiometric equivalence point. This agreement between prediction and experiment validates the model and the values chosen for the parameters. The model also fulfills the requirement that the surface areas of the two leaflets be about the same.

As mentioned in the Introduction, the concept of stoichiometric complexes warrants scrutiny. That sterols associate strongly with phospholipids is manifested by the well known phenomenon of bilayer condensation [33-35]. This association is driven by the shielding of the sterol molecules from water by an umbrella of phospholipid head groups as well as favorable van der Waals interactions, hydrogen bonding and some polar contacts [21, 36]. These sterol-phospholipid associations have often been characterized as arising from an unspecified energy of interaction or an excess Gibbs free energy of solvation such as that proposed to drive the partition of sterols into bilayers [37-41]. However, other analyses support the existence of discrete stoichiometric complexes [21, 33-35, 42-45]. Furthermore, stoichiometric complexes have distinguishing characteristics, illustrated in Figure 1.

(a) Sterol-phospholipid affinity. The relative strength of sterol-phospholipid associations varies with the fatty acyl chains and head groups of the phospholipids as well as with the structure of the sterol [12, 25, 34, 46]. The affinity of phospholipids for cholesterol can exceed that of sterol-binding proteins [47]. How affinity shapes the concentration dependence of the association of sterols and phospholipids is illustrated by curves 3 and 4 in Figure 1. However, attractive interactions also play a role in other models and are therefore not unique to complexes.



F**IGURE 1 |** Stoichiometric complexes in bilayers. Hypothetical isotherms for the association of cholesterol with bilayer phospholipids were generated as previously described [23, 47, 48]. Chemically active (uncomplexed, available) cholesterol is plotted as a function of the bilayer sterol concentration. Curve 4 depicts a bilayer with a relatively high cholesterol/phospholipid affinity and a stoichiometry of C:P = 1:1 such as observed for dipalmitoylphosphatidylcholine and sphingomyelin [25]. Curve 3 has the same C:P = 1:1 stoichiometry as curve 4 but a six-fold weaker affinity; it could represent 1-palmitoyl-2-oleoylphosphatidylcholine [25]. Curve 2 shows competitive displacement of the sterol from complexes; e.g., by hexadecanol [49, 50]. In this example, the stoichiometry of the sterol complex in curve 2 is the same as that in curve 4 (C:P = 1:1) but it has one-thirtieth of its apparent affinity. Curve 1 has a stoichiometry of C:P = 1:2, that reported for dioleoylphosphatidylcholine [25]. Its soft threshold therefore occurs at C/P = 0.50 and its association constant is 30% that of curve 4.

(b) Stoichiometry. Thresholds are observed in the titration by sterols of phospholipid monolayers, liposomes and natural bilayers [15, 21]. That is, sterols interact poorly with probes and ligands at low membrane concentrations but become available at a characteristic turning point. (Such probes include cholesterol oxidase [25, 29], methyl-β-cyclodextrin [51, 52], bacterial

toxins [30, 48, 53] and a variety of membrane proteins [26, 47, 54-56].) These isotherms presumably reflect two states of the sterol: that complexed with phospholipids and the uncomplexed excess that accumulates in the bilayer beyond the stoichiometric equivalence point. The stoichiometries revealed by such thresholds are typically one or two phospholipids per cholesterol [25]. (This generalization must be qualified by the fact that high affinity ligands can pull the sterol from the phospholipids somewhat below their stoichiometric equivalence point and thereby lower the threshold [47, 48].) Curves 1 and 4 in Figure 1 illustrate isotherms for complexes with stoichiometries of C:P = 1:2 and 1:1 respectively.

(c) Competition and displacement. Many intercalating amphiphiles cause an increase in the availability of bilayer cholesterol to probes [49, 50, 57, 58]. They apparently associate with the phospholipids and displace the sterol from complexes mole for mole. A comparison of curves 2 and 4 in Figure 1 illustrates such competitive displacement. In addition, sterol-binding proteins compete with bilayer phospholipids for cholesterol [47, 48].

(d) Uncomplexed cholesterol has a high chemical potential. The thresholds in isotherms like those in Figure 1 are taken to manifest the transition of the sterol in phospholipid membranes from a low to a high chemical activity near their stoichiometric equivalence point [15, 21, 25, 33, 51]. That thresholds in sterol accessibility in fact represent a change in its chemical activity has been demonstrated directly [52].

Cholesterol dependence isotherms such as illustrated in Figure 1 cannot be explained by phase changes since they are observed in monolayers and vesicles containing a single phospholipid above its melting temperature [25, 33, 51]. Nor are the thresholds a manifestation of sterol insolubility or crystallization from a super-saturated bilayer solution because the thresholds in chemical activity occur well below the solubility limit [59]. We conclude that isotherms like those

in Figure 1 do not represent the nonspecific solvation of sterols in phospholipid bilayers; rather, they have the characteristics of chemical complexes.

Other models also have predicted the predominance of the sterol in the outer leaflet based on the high sterol affinity of its exofacial phospholipids [3, 4, 7, 8, 12, 14, 39]. However, there is an important difference between such “affinity mechanisms” and the stoichiometric complex mechanism proposed here, even though the sterol asymmetry in both cases is driven by a preponderance of the same saturated phospholipids in the outer leaflet. In particular, a differential affinity mechanism can impose an asymmetrical transbilayer distribution but it does not set the abundance of the sterol in the membrane. The stoichiometric complex model predicts both the distribution and the concentration of the sterol in the resting plasma membrane bilayer by including the sterol binding capacity of its phospholipids. For the same reason, the stoichiometric complex model can also predict the cross-sectional areas of the two leaflets.

A recent study has proposed a fundamentally different mechanism driving cholesterol to the exofacial leaflet of the plasma membrane [7]. It envisions that there is a substantial deficit in outer leaflet phospholipids and that the consequent gap in its surface area is filled by excess cholesterol. An analysis of this model has found it to be insecure [11].

If, as presumed here, nearly all of the plasma membrane sterols and phospholipids are complexed, the complexes would fill both the phase-separated domains (rafts) and the continuum surrounding them. The complexes would thus contribute significantly to the material properties of the membrane: its condensation, molecular order, thickness, permeability, viscosity, compressibility, deformability, various elasticities, intrinsic curvature and lateral phase heterogeneity [7, 8, 34, 60-64]. Other benefits conferred by the saturation of plasma membrane bilayers with sterols include a reduction of their water permeability (especially important for

osmotically-vulnerable fresh water eukaryotes such as protozoa) and the imposition of a barrier against the entry of potentially deleterious amphiphiles such as xenobiotics [62, 64, 65].

The uncomplexed fraction of plasma membrane cholesterol, normally only a few percent of the total, is chemically active and performs complementary functions [15, 23, 26, 47, 56, 66-68]. It diffuses rapidly across the bilayer so as to equalize the chemical activity of the cholesterol in the two leaflets. The equilibration of the active plasma membrane cholesterol in erythrocytes with plasma (lipo)proteins serves as a homeostatic mechanism that keeps the bilayer phospholipids replete at their stoichiometric equivalence point over months in circulation [7, 24, 27-30]. The cholesterol in the plasma membrane of nucleated cells is similarly buffered by the equilibration of active cholesterol with the plasma (lipo)proteins but is also maintained metabolically at stoichiometric equivalence with the phospholipids [23, 26, 53]. That is, super-stoichiometric cholesterol circulates to the cytoplasm and provides a homeostatic feedback signal to regulatory elements that keep the plasma membrane phospholipids replete with cholesterol while minimizing any excess. Note that intracellular membranes differ from plasma membranes in that their regulatory elements hold their phospholipids well below capacity [23, 26]. The distribution of the sterol in the cytoplasmic membranes then reflects the affinity of their phospholipids rather than their stoichiometry, and the activity of their regulatory elements will vary with the fractional saturation of the membranes with the sterol.

Thus, the stoichiometric equivalence point with the phospholipids is the sterol sweet spot. Less sterol undermines the functions mentioned above while the persistence of a super-stoichiometric excess is toxic, leading to deleterious outcomes such as microcrystal formation [62, 69].

The transbilayer sterol asymmetry inferred for the human erythrocyte might obtain generally. For one thing, the composition and sidedness of the phospholipids in the plasma membranes of different types of nucleated cells are similar to those of red cells [2, 4-7, 9, 10, 70, 71]. For another, the cholesterol concentration in the plasma membranes of nucleated cells, $C_t/P_t \approx 0.7$, is held close to a threshold in their sterol-binding curves that reflects the stoichiometric capacity of their phospholipids [7, 23, 27-30]. These features fulfill the requirement of the model that plasma membrane sterols are maintained at stoichiometric equivalence with the phospholipids.

In conclusion, we anticipate that in any plasma membrane with phospholipids filled to capacity, the cholesterol will favor the leaflet with the higher stoichiometry of its complexes; generally, the exofacial side. (This assumes that the abundance of the phospholipids in the two leaflets is similar.) While current evidence encourages this premise, more detailed data for a variety of plasma membranes are needed.

Authors contributions

TLS and YL contributed to every phase of the work from its conception to its publication.

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Ethics statement

Nothing to report.

Conflict of interest

The authors declare no conflict of interest.

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