Review: A New Concept

Spiral Steroid (SS) Phosphodiesters: role in pregnancy

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Abstract: Spiral steroids (SS) are a newly discovered type of steroid. Originally, they were described as cardiotonic glycosides because they cross-reacted with digoxin-specific antibodies (Digoxin-Like-Materials-DLM). SS differ from the classical steroids in several ways: [a] they are steroid phosphodiester conjugates and displace digoxin from digoxin-specific antibodies, [b] in contrast to all other steroids, cholesterol is not a precursor, [c] SS have more than 21 carbon atoms; the extra atoms form a 5th ring which includes a lactone, [d] SS bind to NaK-ATPase in membranes rather than receptors in the nucleus. SS have been identified by HPLC- mass spectroscopy in extracts from mammals, birds, and oysters but were not detected in plant extracts. Overall, SS seem to be key components in regulating potassium electrolytes during pregnancy and its related diseases – specifically pre-eclampsia and necrotizing enterocolitis.

Keywords: Ionotropin, Kaleotropin, Galotropin, Pre-eclampsia, Spiral Steroids, Necrotizing Enterocolitis, Cardiotonic glycosides, lactone, digoxin-like materials, DLM

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1. Introduction

As of this date, all papers about spiral steroids (SS) were written by IOMA investigator and founder, Fred Chasalow. IOMA LLC was established specifically to commercialize the discovery of steroid phosphodiesters. In brief, IOMA has identified 3 major SS, designated C341, C369, and C381. The first application of SS will be to measure the serum concentrations of the three primary SS to diagnose diseases of pregnancy, specifically, pre-eclampsia and necrotizing enterocolitis.

1.1 Ionotropin – C341 and its precursor C313

Ionotropin (C341) and its three precursors (C313, C337, and C339) were first detected in bovine adrenal extracts [1]. All four of the compounds were phosphocholine steroid diesters. C337, C339, and C341 cross-reacted with digoxin-specific antibodies (DLM). However, C313 was not a DLM. C337, C339 and C341 differ by 2 Da, suggesting they differ by alkene reduction steps. Thus, C337, C339 and C341 would be formed from a Δ5-Δ7 sterol precursor in two steps: first reduction of Δ5-Δ7-sterol by the specific Δ7-8 reductase and, second, reduction of the Δ5-dehydrosterol [2, 3]. Although 5α-DHT is synthesized from testosterone, the specificity for the only known reductase for Δ5-dehydrosterols produces 5β-products, such as cholic acid, digoxin or ouabain. In summary, the four compounds define a biosynthetic pathway with known steps in each biochemical process.

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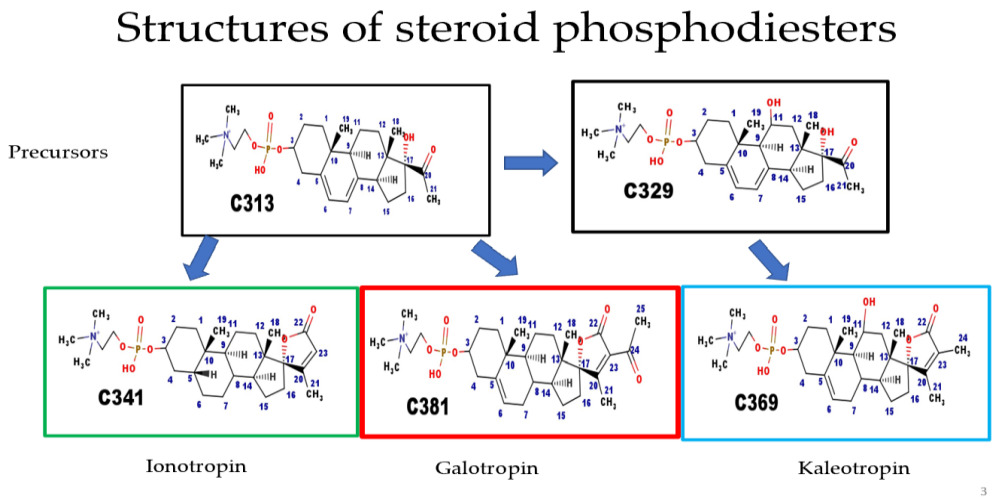

Fig. 1. Proposed structures for the 5 major steroid phosphocholine diesters.
The chemical composition of the steroid fragment of the phosphodiester compounds was determined by trial-and-error analysis using the mass determined by LC-MS [4]. Although there is no inherent requirement, only one molecular composition satisfied the m/z for each molecule. This does not eliminate isomers.

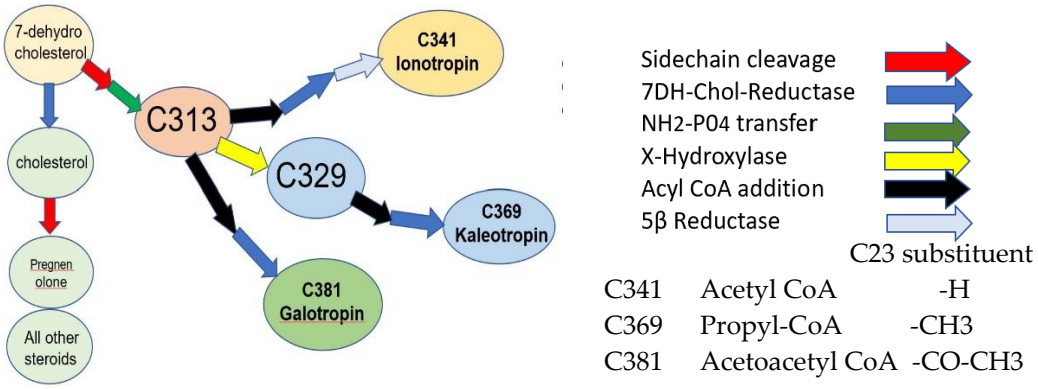

Fig. 2. Enzyme steps for the endogenous synthesis of SS.
In contrast to Hamlyn's proposed biosynthesis of Endogenous Ouabain, for each of the steps identified in the figure, the color-coded arrows connect substrates and products that have been identified by LC-MS. This satisfies Nicholls requirement for an endogenous biosynthetic pathway.

Table 1 Name and localization of Spiral Steroids (SS)

Name	Symbol	# of C atoms	Tissue target
Ionotropin	**C341**	23	heart and kidney
Kaleotropin	**C369**	24	gonads, placenta and during pregnancy
Galotropin	**C381**	25	colostrum and post-partum milk

SS have a lactone E-ring and displace digoxin from digoxin-specific antibodies.

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Table 2 Example: 5 peak pattern for C313

	m/z		
	184	Phosphocholine fragment – base ion	
A]	313	M-17	Loss of hydroxy group from steroid
B]	496	M+1	Hydrogen ion
C]	518	M+ 23	Sodium ion
D]	534	M+39	Potassium ion
E]	459	M+23-59	Loss of trimethylamine from Sodium ion

Blood from readily available mammalian species (goats, sheep, cattle, rats, mice, rabbits, horses, and pigs) was obtained, extracted with acetonitrile and analyzed by LC-MS. As sodium ions are the main electrolyte in blood, the main ions in the extracts were Na+ ions, with much smaller amounts of K+ ions. Thus, a single steroid phosphodiester generates a complex LC-MS spectrum, including 5 different ions. As shown in Fig. 1, there are at least 5 different parent compounds. Thus, a typical LC-MS spectrum has more than 20 ions that derive from only 5 unique steroids [4].

Blood from chickens and turkeys was obtained and analyzed by LC-MS. C313 was a major component, just as it was with the mammalian samples. However, C341 was not detected but the ions for C339 were present. This may indicate that these avian don't have the enzyme necessary to reduce the Δ5-6 alkene. Note that avian species do not use DHEA or DHEA-S as a weak androgen. Thus, in contrast to the human weak androgen, which is DHEA-S, there is no need for the binding site on NaK-ATPase to distinguish the SS from the weak androgen, DHEA-S.

2.0 Spiral Steroids (SS) vs. Endogenous Ouabain (EO)

In 1991, from 80 L of plasma, Hamlyn's team isolated 13 μg of a cardiotonic glycoside. At first, each step was monitored by an RIA specific for digoxin [5]. They claimed, on the basis of an NMR study, that the isolated material closely resembled ouabain. In later studies, they developed an assay with specificity for ouabain. Manunta used the assay to evaluate the role of endogenous ouabain in cardiovascular function [6]. Buckalew evaluated the role of endogenous ouabain in severe pre-eclampsia [7]. Bagrov investigated novel therapeutic target for endogenous ouabain [8]. Altogether, there are over 1000 publications in which, investigators have used an immunoassay for ouabain to monitor cardiotonic glycosides to diagnose human disease. In 2014, despite this mass of data, Blaustein wonders, "Why isn't endogenous ouabain more widely accepted?" [9]

In 1997, Fedorova's team isolated a marinobufagenin-like compound from human samples [10]. An immunoassay for marinobufagenin was developed and has also been used for diagnosis [11, 12]. For comparison, ouabain and digoxin are plant glycosides while marinobufagenin can be isolated from toad skin, but only after extensive methanolysis. The endogenous parent compound for marinobufagenin doesn't seem to be known.

In response to Blaustein, Nicholls asks, is " Ouabain: a new steroid hormone?"[13] Nicholls is concerned because, except for immunoassays, neither ouabain nor marinobufagenin, can be detected by any sensitive instrumental method of analysis, including LC-MS [14, 15]. Further, Hamlyn has not been able to describe a biosynthetic pathway utilizing well-characterized enzymes that might be present in mammals [16]. In contrast, Fig. 2 shows a proposed scheme for biosynthesis of SS compounds and SS compounds can be detected by LC-MS from many different species. In summary, SS satisfy Nicholls' test for an endogenous cardiotonic steroid.

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The investigation of endogenous ouabain needs to be carefully re-evaluated [17]. There are at least three NaK-ATPase isomers and there are at least three candidates for the corresponding endogenous cardiotonic glycoside [18]. It remains unclear if there is a one-to-one correlation between SS isomers with specific endogenous ouabain-like compounds matching with specific isomers of NaK-ATPase [19, 20, 21].

2.1 Tissue specific Spiral Steroids

Most steroids function by binding to high affinity nuclear receptors. Serum levels of Ionotropin are about 1 mg/L, which is equivalent to 2,000 nM [1]. For comparison, normal testosterone serum levels are 5-40 nM. Thus, Ionotropin (C341) could not function via nuclear receptors because the receptor would be saturated at all times. Note that patients with Kaleotropin (C369) in their serum do not have hypertension [3] even though it is a Spiral Steroid.

2.2 Tissue specific NaK-ATPase as a signal transducer

SS stimulate NaK-ATPase, pumping K+ into cells. The specific SS must match the specific isozyme of the NaK-ATPase α- subunit [18, 19, 20, 21, 22]. If K+ is inadequate, SS precursors are synthesized, converted to a specific SS and, in due course, in the specific tissue, stimulates increased K+ transport [23].

2.3 Discovery

The discovery of SS started with investigation of the role of breast cysts in breast cancer [24]. Human breast cyst fluids were divided into two types: Type 1, with high K+, low Na+ electrolyte levels, just like milk, and Type 2 with the reverse electrolyte pattern, similar to blood plasma. The oncologists had two questions: question #1: was Type 1 cyst fluids a precursor or risk factor for breast cancer? and, question #2: how did the K+ get into Type 1 fluids against the gradient?

Answer #1: The incidence of breast cancer in women who had breast cysts was not affected by the electrolyte type of their cyst.

Answer #2: The investigators established a team to collect breast cyst fluids. Each sample was analyzed for electrolytes, DHEA-S-like material and DLM. Although only a small volume of fluids was obtained from each cyst, the concentration of DLM in the Type 1 fluids was very high, typically 0.6 ngE/ml. For comparison, blood plasma has less than 0.02 ngE/ml. In due course, based on the high concentration of DLM in the Type 1 fluids, methods were developed to isolate SS. The SS present in the breast cyst fluids is different than the SS isolated from bovine adrenals. Based on LC-MS fragmentation, as shown in Table 1, the SS compound in milk was identified as C381 (Galotropin) and that in adrenals was identified as C341 (Ionotropin) [1].

3.0 SS during gestation

Fetal nutrition is provided through the placenta. Plasma electrolytes are 145 mM Na+ and 3-5 mM K+. Intracellular K+ levels are ~100 mM. To get K+ into cells, it must be 'pumped' against the gradient. As the size of the fetus increases, to maintain perfusion, the fetal blood pressure must increase, but the regulatory mechanism was unknown. During the 3rd trimester, there are two aldosterone-signaling defects [25]. First, maternal aldosterone synthesis is reduced and, second, endothelial sodium channels (ENaC) are blocked. Together, this leads to Na+ "wasting." The 'wasted' Na+ becomes the amniotic fluid.

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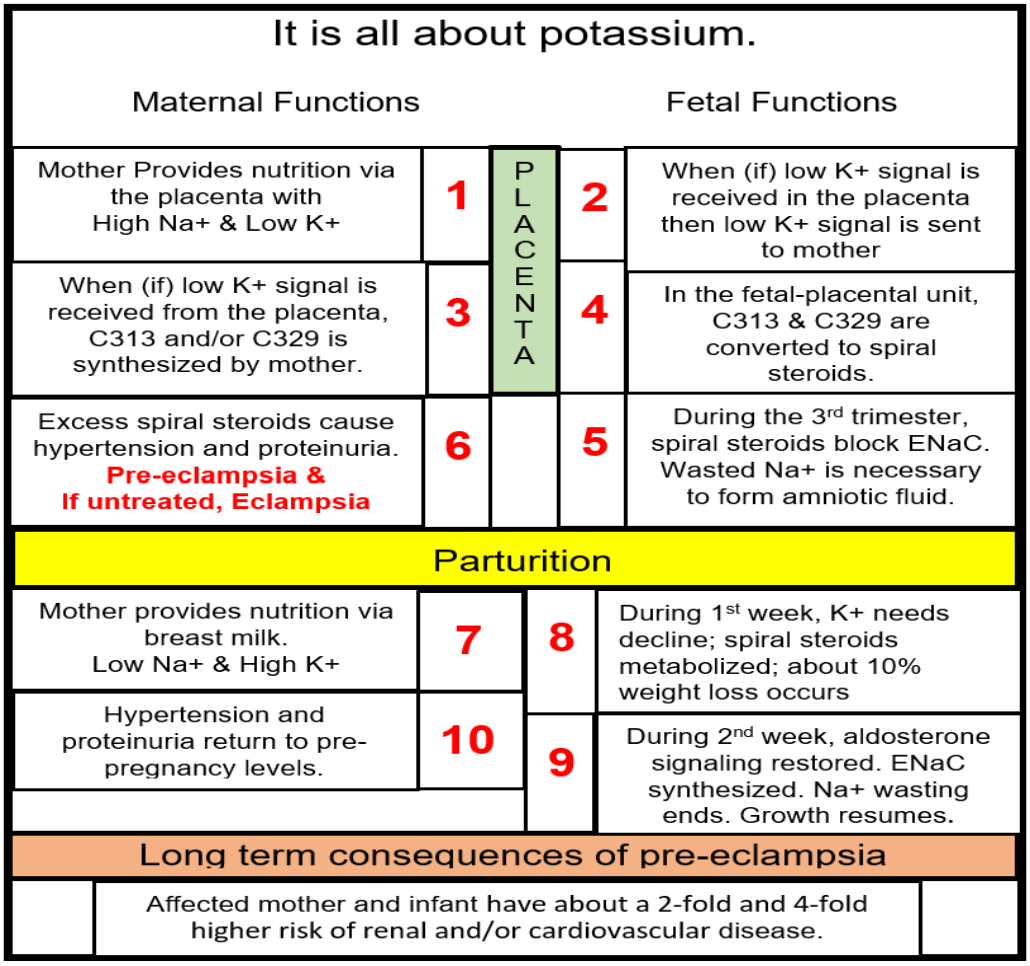

Fig. 3 Changes in electrolyte regulation during pregnancy.
This chart illustrates the changes in potassium electrolytes that occur during pregnancy [26].

3.1 It's all about potassium

Fig. 3 shows the changes in electrolytes that occur during pregnancy and the corresponding changes in the SS. The biological processes were known but the role of the SS was unrecognized. Coincidentally with the onset of aldosterone insensitivity, elevated levels of SS begin during the third trimester [25]. The SS that stimulates K+ accumulation in the fetus (Kaleotropin) is not the same SS that causes maternal hypertension (Ionotropin) [3].

3.2 Timeline for diseases of pregnancy

Fig 4. Shows the proposed timeline for the development of pregnancy related diseases. The integration of the role of the SS suggests pre-eclampsia is a disease rather than a syndrome with a collection of symptoms. The proposed biochemical basis is the biosynthesis of Ionotropin from the C313 precursor rather than the synthesis of Kaleotropin.

NEC has been visualized as a disease of infancy, rather than a fetal disease. Galotropin is necessary to transport K+ from plasma to milk and colostrum. Milk comes 'in' during the last month of pregnancy. Thus, milk from mothers with premature babies born by C-Section does not have Galotropin and is likely to have inadequate K+ levels.

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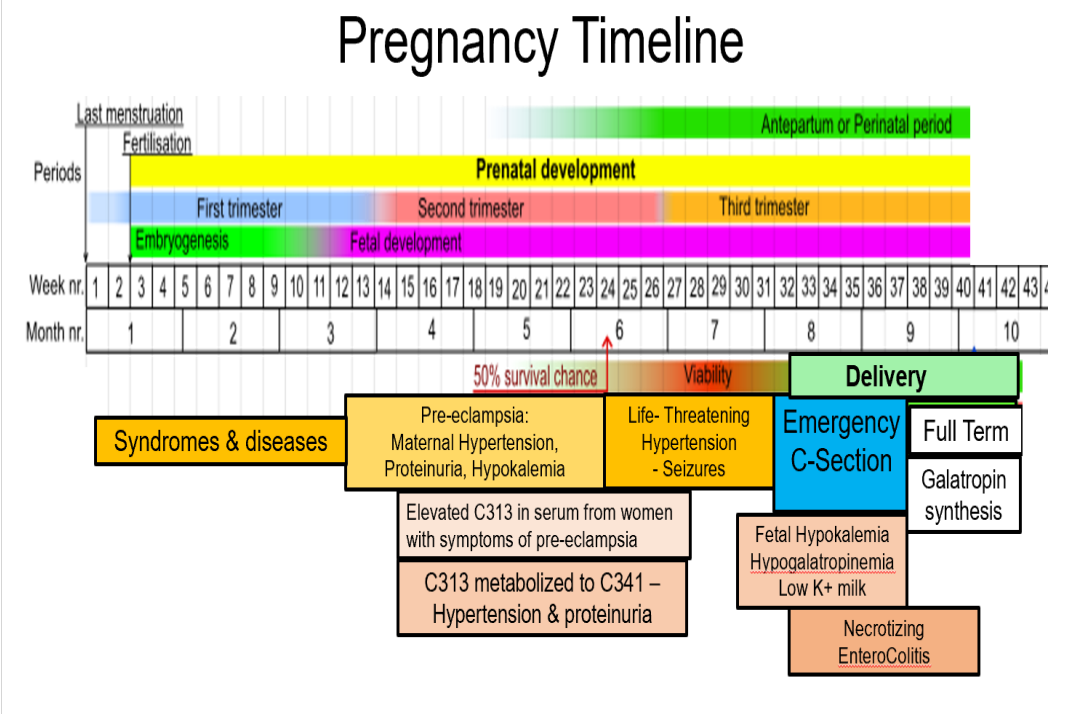

Fig. 4. Timeline for syndromes during pregnancy.
Until the discovery of SS, pre-eclampsia and necrotizing enterocolitis were considered syndromes with an unknown cause. The "pilot" study (Fig.5) showed that the C341 precursor, C313, was elevated in 65% of patients with pre-eclampsia ($P<0.05$) [27]. No other proposed diagnostic hormone measurement is statistically significant. Elevated C341 causes hypertension, just as is observed in pregnant women with pre-eclampsia.

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4. **C313 & C329: SS precursors: Diagnosis of Pre-eclampsia:**

As illustrated in Fig. 5, maternal serum levels of C313 and C329 can be used as a monitor of hypertension and pre-eclampsia. High levels of C313 lead to increased synthesis of C341 and lead to hypertension. In contrast high levels of C329 would indicate that the fetus was making C369, which stimulates neonatal K+ accumulation and restores fetal growth.

There are several significant points in this data. First, the Z-score data of normotensive pregnant women when compared to the Z-score data of women with pre-eclampsia clearly shows significant differences. This is the first example of a serum biochemical marker that distinguishes women with symptoms of pre-eclampsia from normotensive pregnant women. Second, 12 of 20 samples had Z-scores >2 for at least one of the precursors, whereas only 1 of the samples from normotensive women had Z-score >2. That result could be the basis for an early diagnostic marker for pre-eclampsia or for a progression marker to evaluate therapeutic interventions. Third, C341 levels, detected at m/z=546 Da, were also elevated in the women with pre-eclampsia (compare peak height of m/z=546 Da to the corresponding internal control at m/z=430 Da, see lower panels, Fig.5). Elevated levels of C313 would lead to synthesis of C341 and lead to hypertension. Finally, maternal hypokalemia, a common observation in pre-eclampsia, would lead to inadequate K+ in colostrum and lead to neonatal growth failure [27].

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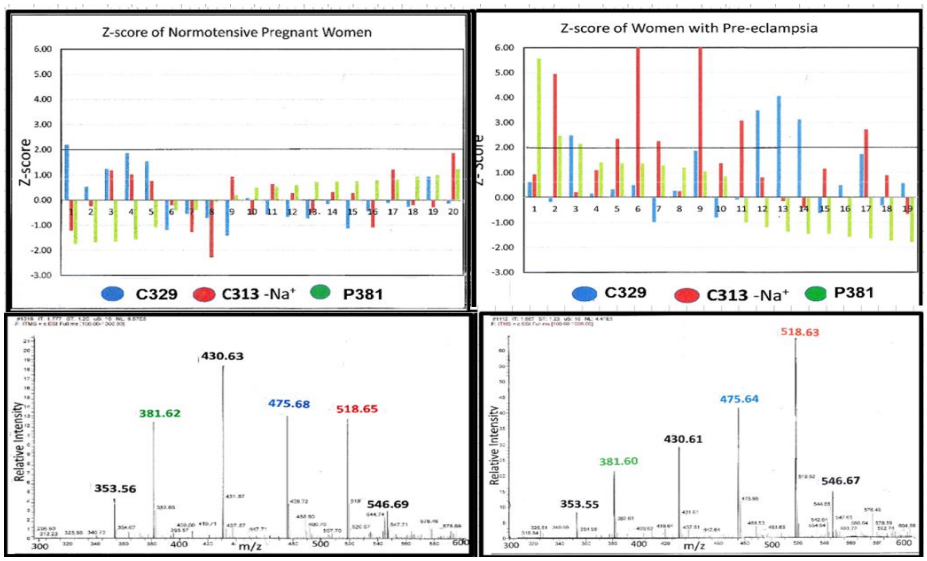

Chasalow F, John C, & Bochner R. (2019) Spiral steroids as potential markers for pre-eclampsia: a pilot study. *Steroids*. Nov. 151:108466. DOI:10.1016/j.steroids.2019.108466 [27].

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Fig. 5. Steroid phosphodiesters during the second trimester.

Serum samples (22-24 weeks of gestational age; n=20 normotensive women; n=20 women with pre-eclampsia) were obtained from Global Alliance for the Prevention of Prematurity and Stillbirth (GAPPS).

Left column: normotensive women;
Right column: women with pre-eclampsia
Top: Z-scores based on intensity of miltefosine as internal control.
Bottom: Representative mass spectra

Method of analysis: For each spectrum, the intensity of each ion was compared to the intensity of the ion generated by miltefosine (hexadecyl-phosphocholine). The mean and standard deviations of ions from the normotensive women were calculated and used to generate Z-scores for each of the 40 samples. The Z-scores for each sample were graphed as a cluster. The clusters fall into three patterns:

Pattern A: 2, 6, 7, 9, 11, 17	C313 Z score >2
Pattern B: 3, 9, 12, 13, 14, 17	C329 Z score >2
Pattern C: 1, 4, 5, 8, 10, 12, 15, 16, 18, 19	No Z score pattern

Table 3. Ions detected by LC-MS in serum from pregnant women

Symbol	Description			NaK-ATPase isoform
C313	Ionotropin precursor –	C518 - Na+	P	
C341	Ionotropin	C546 - Na+	SS	Kidney, heart
C329	Kaleotropin precursor	C534 – Na+	P	
C475	Kaleotropin precursor	C534 – Na+	P	
C353	Kalotropin Intermediate	C358 - Na+	SS	
C369	Kalotropin	C574 - Na+	SS	Gonad, Placenta
C381	Galotropin	C586 – Na+	SS	Mammary gland, milk Neonatal GI tract (?)

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Red labelled ions were detected in serum from pregnant women at 22 weeks of gestation.

5.0 SS in gestational hypokalemia

SS are an important element in each stage. At each stage, the fetus has hypokalemia and synthesizes a specific SS as a messenger to restore fetal and/or neonatal K+ levels.

1. Inadequate implantation – possibly caused by local hypokalemia
2. Pre-eclampsia – Hypertension & proteinuria (see Fig. 5)
3. Emergency C-Section -Ionotropin-emia- life-threatening hypertension and seizures
4. Lack of Galotropin in colostrum and milk -
5. Long term risk of cardiovascular and renal disease – toxicity of SS during pregnancy

5.1 Inadequate implantation.

The central hypothesis is that pre-eclampsia results from defective artery remodeling caused by an imbalance of antiangiogenic factors [28, 29]. The result of the process is inadequate fetal nutrition, specifically hypokalemia.

5.2 Pre-eclampsia – hypertension and proteinuria

These symptoms are caused by maternal efforts to provide K+ to the fetus [26]. If K+ levels are insufficient, the mother responds by making the SS precursor, C313 (see Fig. 3). C313 is the branch point for the fetal-placental unit biosynthesis both of C341 and of C369. As obligate heterozygotes for 7-dehydrosterol reductase deficiency (SLO syndrome) all have high serum levels of C369 but do not have hypertension, the hypertension must be caused by C341, rather than by C369 [3]. Digibind therapy of patients provided a modest short-term decrease in their hypertension [30]. As C341 was isolated on the basis of its cross-reactivity with the same digoxin-specific antibody, this seems to confirm C341 as the cause of the hypertension in pre-eclampsia [31].

5.3 Life threatening hypertension and seizures

About 50,000 pregnant women every year develop life threatening hypertension and/or seizures [32, 33]. Therapy includes anti-hypertensive drugs but these fail to maintain normal blood pressure. The only successful therapy is immediate C-section [34]. C-section typically occurs during the 7th or 8th month, when an infant typically weighs 1.5 to 3 kgs.

5.4 Galotropin in colostrum and milk

The electrolyte composition of colostrum and milk differs significantly from that of blood plasma. Colostrum and milk are high K+ and low Na+ while blood plasma is high Na+ and low K+. Galotropin, C381, was isolated from the Type 1 breast cyst fluids but it was not present in the Type 2 fluids [35]. Prior to the discovery of the SS, there was no explanation for the mechanism that accumulated K+ against the concentration gradient and how the process was regulated.

5.5 Long term risk of cardiovascular and renal disease – possible cause: hypokalemia

After parturition, infants are fed by nursing. At full term, human milk contains 60-100 mM K+ and 10-20 mM Na+ [36, 37]. Full-term milk (and milk from goats, sheep and cows) contains C381, Galotropin. Galotropin serves two functions: [i] stimulating K+ accumulation in colostrum and milk and [ii] stimulating K+ absorbance in the neonatal gastrointestinal tract. However, despite normal levels of aldosterone, newborn infants are Na+ wasting and lose about 10% of their weight during the first week post-partum. The end

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of the weight loss occurs at about two weeks of age and coincides with the disappearance of SS from the neonatal circulation [3].

6. Necrotizing Entero Colitis (NEC)

C-section generally ends the risk of seizures for the mother. However, the infant typically weighs less than 3 pounds, has not yet matured enough to suckle, and, until the baby learns to suck effectively, is often fed by gavage and/or tube feedings. Infant formula is specially pasteurized to assure lack of contamination. Normally, Galotropin is not synthesized until the 9th month of gestation, long after C-section. However, whether or not Galotropin was synthesized by the mother and is stable to the specific pasteurization process has not been confirmed. One way or the other, infants who have been delivered by early C-section to avoid hypertension and seizures will have low K+ levels. When nursed to an infant, the milk would be K+ deficient and without Galotropin, the NaK-ATPase in the GI tract could not import K+. The GI tract fails to grow, preventing maturation of the GI tract and leading to growth failure of the GI tract and lack of resistance to infection.

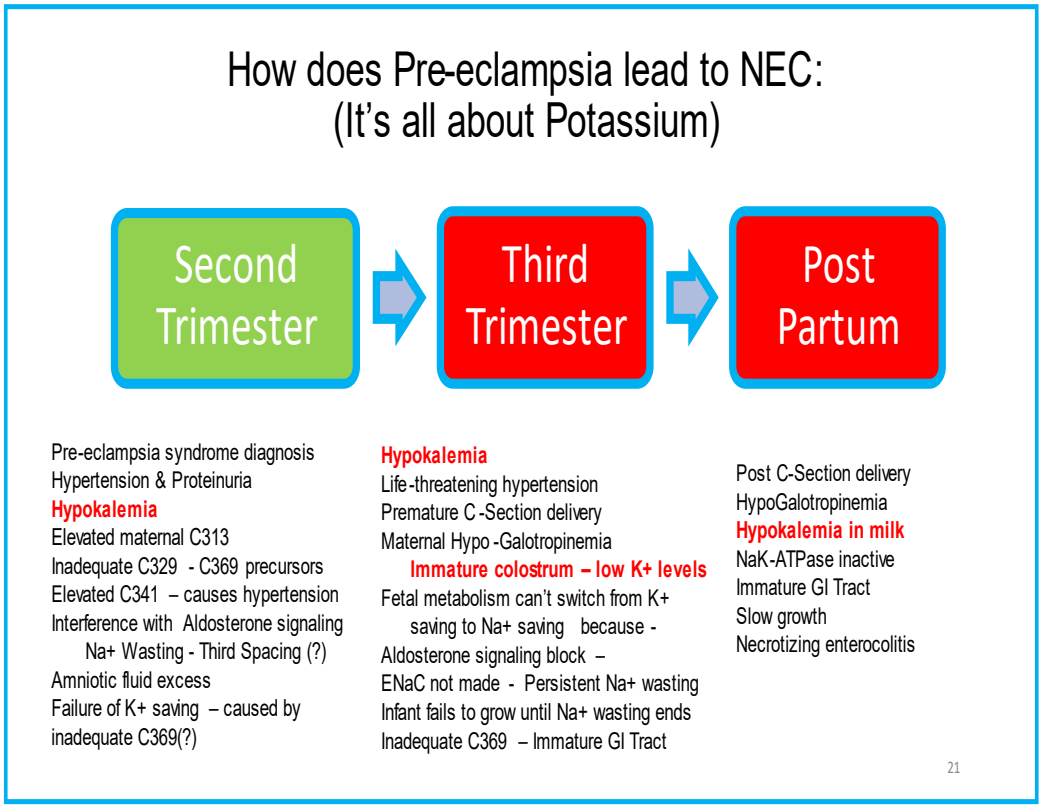

Fig.6. Schematic showing how pre-eclampsia and hypogalotropinemia could lead to necrotizing enterocolitis (NEC). [38-42]

7. Proposed prevention and therapy of NEC

Therapy has to begin with reversing the hypokalemia typical of pre-eclampsia.

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Institutional Review Board Statement:

This paper is a review of publications from many laboratories. The authors of each cited publication complied with Institutional Review Board requirements appropriate at the time of original publication.

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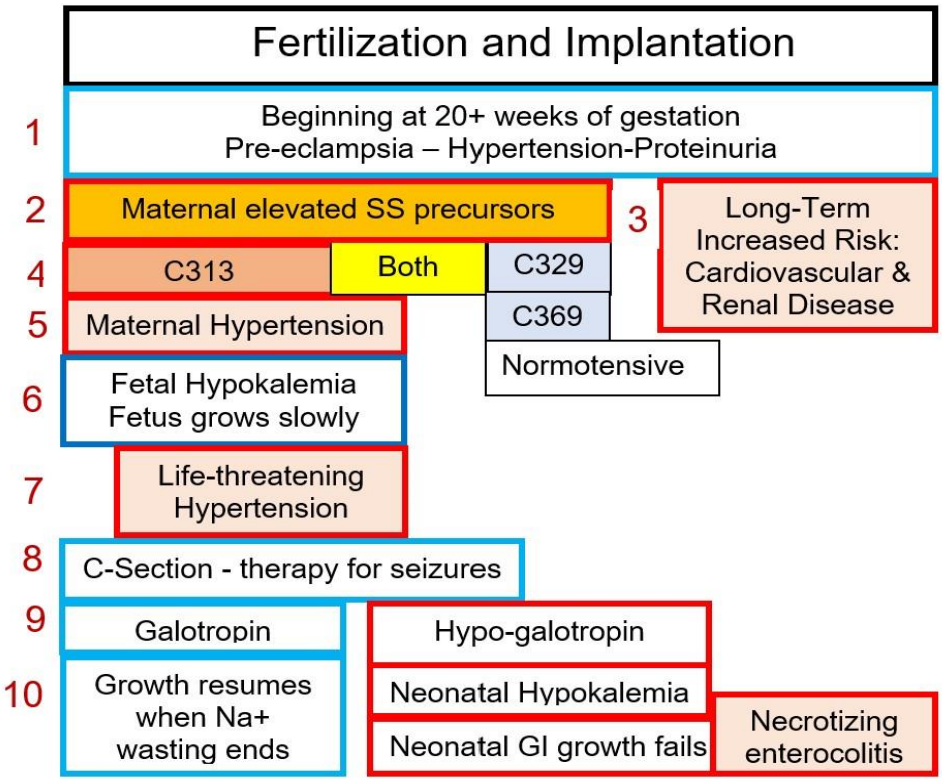
APPENDIX: Figure 7



Steps leading from Pre-eclampsia to Necrotizing Enterocolitis

Line Explanation

1. Pre-eclampsia is a syndrome characterized by hypertension and proteinuria It begins as early as 20 weeks of gestation.
2. There is no FDA approved diagnostic for pre-eclampsia. In a pilot study, we have shown that SS precursors are elevated in many patients with a diagnosis of pre-eclampsia. C313 and C329 are the precursors for Ionotropin (C341) and Kaleotropin (C369). Elevated C341 levels are associated with hypertension.
3. In contrast, C369 is elevated in patients who are obligate heterozygotes for 7-dehydrosterol reductase deficiency. These patients do not have hypertension. Thus, gestational hypertension is caused by C341.
6. Affected fetuses develop hypokalemia and can only grow slowly.
7. Many affected patients develop life-threatening hypertension.
8. The physician treats this with immediate c-section.
9. Depending on the gestational status, the patients may have secreted Galotropin. In which case, milk will have adequate K+ and the neonate will begin to grow.
10. If at the time of the C-section, the neonate was too immature to synthesize Galotropin, then the neonate will continue to have hypokalemia and will be at risk for necrotizing enterocolitis