Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1//2009- 12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814-935-1081

5. **Grant SAP Number:** # 4100047645

6. **Project Number and Title of Research Project:** 31 - Autism Indicators: Erythrocyte Membrane Fluidity and/or Lipid Composition

7. **Start and End Date of Research Project:** 07/08/2009 – 05/31/2011

8. **Name of Principal Investigator for the Research Project:** Cara-Lynne Schengrund, PhD

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ $77,747

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Ali-Rahmani	Graduate Assistant	100% Yr 1	30,216.84
Lin	Manager NMR core/ Asst. Professor	27.5% ½ Yr 1	$20,680

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Schengrund	Professor emeritus	3
Ramer	Professor	2

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. **Co-funding of Research Project during Health Research Grant Award Period.** Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____√_____

If yes, please indicate the source and amount of other funds:

11. **Leveraging of Additional Funds**

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No___√_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____ No____√_____

If yes, please describe your plans:

12. Future of Research Project. What are the future plans for this research project?

Our results support the concept that changes in lipid composition may contribute to the autistic phenotype. They also point out the need for additional research to ascertain whether children with autism exhibit changes in expression of proteins involved in cholesterol uptake/release by cells, the expression of enzymes involved in glycosphingolipid metabolism, and whether changes anticipated based on the above results affect expression of proteins known to affect behavior. As more information about the expression and underlying metabolism of cholesterol and glycosphingolipids, major lipid components of membrane associated lipid rafts, by individuals affected with different neurological problems becomes known it should become possible to identify their specific effects. The observations presented point out the need for further investigation of lipid metabolism in autistic subjects the results of which could provide insights into development of new treatment strategies for autism spectrum disorders.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____√_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian			1	
Other				
Unknown				
Total			**1**	

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes_____ No____√_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes____X_____ No_____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

    This project strengthened the institution's basic science research base for autism studies and facilitated a new translational research collaboration between investigators in the Department of Biochemistry and Molecular Biology and the Department of Psychiatry.

16. **Collaboration, business and community involvement.**

    16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____√_____ No_____

If yes, please describe the collaborations:

Dr. Xianlin Han, then at Washington Univ., St. Louis, did the initial lipidomic assays. He is currently at the Sanford-Burnham Medical Research Institute in Orlando, FL.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___√_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____√_____

If yes, please describe involvement with community groups that resulted from the research project:

17. **Progress in Achieving Research Goals, Objectives and Aims.**
List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims for the entire grant award period. Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.
Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance

Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project goals, objectives and specific aims

Autism is defined by an individual's behavior and is one of several pervasive developmental disorders. According to NINDS, experts estimate that three to six children out of every 1,000 will have autism, with males four times more likely than females to be affected [1]. Children with autism have impaired social interaction, abnormal communication skills, and repetitive behaviors or interests. It is these behaviors that are used to identify children with autism [usually as set forth in 2]. Because the behaviors may be subtle, or overlap with typical development in infants and toddlers, it is often not diagnosed until a child is three years of age or older. Delay in diagnosis is problematic as the earlier autism is identified and treatment started, the more likely that improvement will be noticed.

Analysis of a number of factors including genetic, metabolic and environmental has failed to identify the primary cause for autism. Studies focused on lipids present in the blood of affected children have given mixed results. A report by Bu et al [3] on the fatty acids present in erythrocyte membranes obtained from autistic subjects and controls did not support the hypothesis that a defect in fatty acid metabolism was the underlying cause of autism. These observations contradicted those in an earlier paper that reported the fatty acid composition of erythrocyte phospholipids obtained from a single patient contained more highly unsaturated fatty acids than controls [4]. The apparent difference in results emphasizes the need to study multiple samples to determine whether there is a consistent difference regardless of the conditions under which samples are obtained.

Evidence for alterations in cholesterol metabolism in at least a subset of children with autism spectrum disorders is accumulating. Kelley [5] found that patients with Smith-Lemli-Opitz syndrome (SLOS) had elevated levels of 7-dehydrocholesterol, the immediate precursor in the synthesis of cholesterol, due to a deficiency in 7-dehydrocholesterol reductase [6], the enzyme that catalyzes reduction of 7-dehydrocholesterol to cholesterol. Because about 50% of SLOS patients meet the criteria for autism the finding of elevated 7-dehydrocholesterol was hypothesized to serve as a possible model for understanding the genetic causes of autism and the role of cholesterol in autism spectrum disorder [7]. Research by Tierney et al [8] in which serum samples from 100 children with autism were studied indicated that 19 of the samples had cholesterol levels below the 5th percentile for children over 2 years of age. If the level of free cholesterol found in cell membranes is reduced that could affect development and possibly formation of lipid rafts – areas of cell membranes known to be enriched in cholesterol, sphingolipids, and signal transduction molecules. Cholesterol is essential for myelination [9] and research has indicated that low levels of cholesterol may contribute to development of neuronal problems [10].

In previous work we observed that acute disruption of membrane cholesterol using either methyl-ß-cyclodextrin (MβCD, sequesters membrane cholesterol and extracts it [11]) or filipin (aggregates membrane cholesterol [12]) was accompanied by increased expression of GM1 (13). Increased levels of GM1 have been found in cerebral spinal fluid from children with autism spectrum disorders (14) and increased serum levels of anti-GM1 auto-antibodies were found in children diagnosed with autism (15). Since both cholesterol and GM1 are found in lipid rafts alterations in their content could result in altered signal transduction.

These observations led us to hypothesize that the lipid composition of membranes isolated from red blood cells obtained from children diagnosed as having autism would differ from those from normally developing, age, gender, and location-matched controls. It was anticipated that if a difference was observed it might be possible to 1) develop a simple diagnostic test to identify children with autism and 2) determine what cell function(s) the changes affected and whether they contributed to the problem. The question was approached by determining 1) anisotropy, 2) phospholipid composition, 3) cholesterol, and 4) GM1 content of red blood cell membranes isolated from children with autism and comparing them to those obtained for comparison children.

Methods and findings

Approval for study of not more than 20 children diagnosed as having autism and 20 controls was obtained from the Human Subjects Protection Office (IRB Protocol # 30838EP) prior to initiation of this study. Children enrolled in the study were evaluated for symptoms of autism using a two-step procedure. Initially each potential enrollee was evaluated in the pediatric autism clinic by an experienced developmental pediatrician. Individuals that met DSM-IV-R criteria to have autism, had their diagnosis confirmed using an interview specialized for research use, the Autism Diagnostic Interview-Revised (ADI-R [2]). Scores obtained from this interview were used to grade severity (see Table 1, AD score). Comparison children (controls) were evaluated using the Modified Checklist for Autism in Toddlers (M-CHAT) for children 3-5 years 11 months and the Social Responsiveness Scale for children 6 -9 years completed by a parent. These standardized questionnaires were utilized in order to exclude children with symptoms of autism or other developmental disorders from being included in the control group. The children in both groups ranged in age from 3 to 8 years.

Autism samples			Controls	
Age	Sex	AD score	Age	Sex
3*	M	53	3*	M
3*	M	31	3	F
3	M	33	4	M
4*	M	30	4*	F
4*	M	45	5*	F
4	M	38	5*	F
5*	M	39	6*	M
5	M	44	6*	M
6*	M	41	6	F
6*	M	49	6	F
6*	M	36	6	F
6*	M	38	7*	M
6*	M	44	7*	M
6	F	NA	7	M
7*	M	34	7*	F
8*	M	44	7	F
			8*	M
			8*	F
			8	F
			8	F

Table 1: Age, sex, and AD score (when applicable) of children from whom samples were obtained for analysis.

Three ml of blood, drawn in tubes with zinc free stoppers/no anticoagulant, were centrifuged to remove serum and pellets taken for analyses. Erythrocytes (RBCs), dispersed by addition of an equal volume of balanced salt solution (pH7.6) to each pellet, were isolated by centrifugation on

Ficoll-Paque™ Premium (GE Healthcare, Piscataway, NJ) at 400xG for 30 min at room temperature. Erythrocytes, recovered at the bottom of the tube, were washed (20mM Tris-HCl containing 0.13M NaCl, pH 7.4) prior to lysis in cold 5mM sodium phosphate, pH8.0. Membranes were recovered by centrifugation (14,000xG, 4˚C) and washed until hemoglobin free. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Samples were then stored at -80˚C until used.

Anisotropy measurements were done on the isolated RBC membranes in order to obtain a first approximation of whether a difference in membrane fluidity was associated with autism. The hypothesis that it might be was based on the report of elevated levels of amino-glycerophospholipids in RBCs and plasma from children with autism compared to controls (16) and that fluorescence anisotropy measurements of RBC membranes isolated from the blood of autistic children indicated they were less fluid than RBC membranes isolated from blood of typically developing peers (17). Anisotropy experiments were done as follows: tetrahydrofuran containing 2mM 1,6-diphenyl-1,3,5-hexatriene was pipetted into a 1000-fold volume of phosphate buffered saline, pH7.4 and stirred to allow evaporation of the tetrahydrofuran. Sample containing ~0.1mg of protein/ml of phosphate buffered saline was then diluted with an equivalent volume of the fluor. To allow the fluor to interact with the protein, samples were incubated at 37˚C for two hours. Anisotropy measurements were then made using an ISS PC1™ spectrofluorimeter using an excitation wavelength of 365nm and a 400nm Ealing colored glass filter for emitted light (λ_{max} for the fluor is 435nm, a wavelength transmitted by the filter).

It can be seen (Figure 1) that the results obtained for RBC membranes isolated from 11 samples from children with autism did not differ from samples obtained from 14 controls. Interestingly, results were similar whether samples were analyzed within 24hrs of preparation or after storage at -80˚C for several weeks. While these results were not anticipated, they did not eliminate the possibility of specific lipid changes that when combined would have no effect on overall membrane fluidity.

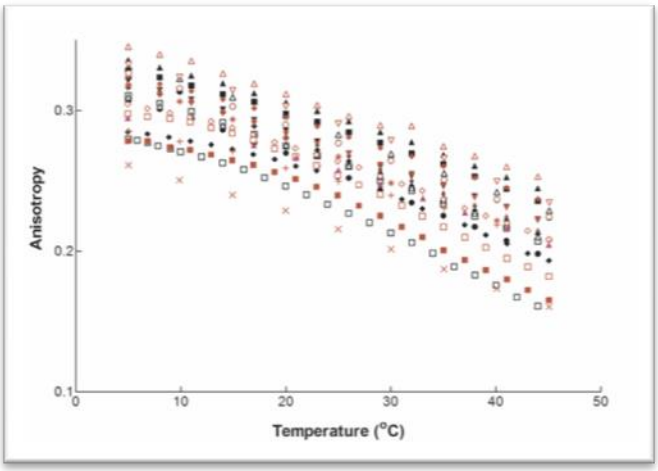

Figure 1: Anisotropy data for RBC membranes isolated from 11 children diagnosed with autism (black) and 14 comparison (red) children. Note that in several cases the symbols overlap.

Phospholipid analyses were done using lipids extracted from isolated RBC membranes containing 1mg of protein using shotgun lipidomics [electrospray ionization mass spectrometric analysis (13)]. Phospholipid standards (Avanti) were prepared using phospholipids having fatty acid compositions not found *in vivo*. Samples containing 1 mg of protein were added to a test tube containing dried phospholipid standards and extracted using chloroform-methanol-0.05M LiCl (protocol obtained from Dr. X. Han, Washington Univ. School of Medicine, St. Louis,

MO). Phospholipid standards provided the internal standard for quantification of each of the phospholipids analyzed. Six samples were analyzed by Dr. Han. Analysis of the data indicated no significant differences between the ratios of the different classes of phospholipids (Table 2), nor in the fatty acid composition of those isolated from membranes isolated from blood from children with autism and controls (data not shown). Failure to see any differences dampened Dr. Han's enthusiasm for continuing the collaboration and he indicated that any further assays would cost $2000 a piece. Without intriguing preliminary data this was not pursued.

Table 2: Phospholipid composition of erythrocyte membranes isolated from four children diagnosed with autism (AO) and two comparison (CO) children.

Phospholipid	AO	AO	AO	AO	CO	CO
PC	88	82	135	76	101	87
PE	342	288	572	280	380	352
PS	200	164	359	197	216	191
SM	158	171	278	154	179	171
PC:PE	0.25	0.28	0.24	0.27	0.27	0.25
PC:PS	0.44	0.5	0.38	0.38	0.47	0.46
PC:SM	0.55	0.48	0.48	0.49	0.57	0.51
PE:PS	1.7	1.8	1.6	1.4	1.8	1.8
PE:SM	2.2	1.7	2.1	1.8	2.1	2.1
PS:SM	1.3	1.0	1.3	1.3	1.2	1.1

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; and SM, sphingomyelin

Actually, these results agreed with a report indicating that analysis of the fatty acids present in erythrocyte membrane lipids obtained from autistic subjects and controls did not provide "strong evidence for the hypothesis that abnormal fatty acid metabolism plays a role in the pathogenesis of autism spectrum disorder." (3). Interestingly, another paper suggested that the fatty acid composition of erythrocyte phospholipids contained more highly unsaturated fatty acids than controls (4). Differences could reflect diet (19) or whether samples were obtained from children who had been fasted for a fixed length of time prior to the time blood was taken. These possibilities emphasize the need to study multiple samples to determine whether there is a consistent difference regardless of conditions under which samples are obtained.

The *possibility of using NMR* to obtain an indication of the relative amounts of the different classes of phospholipids present in the membranes was investigated by Dr. Lin under the direction of Dr. Fang Tian. Two sample preparations were compared: solubilization of membrane components using sodium dodecyl sulfate (SDS) and extraction of the lipids with chloroform-methanol. Better spectral resolution was obtained when lipids were extracted with chloroform-methanol prior to analysis using ^{31}P and ^{1}H NMR. Phosphatidylcholine (PC) and sphingomyelin (SM) were readily identified by NMR while characterization of phosphatidylserine (PS) and phosphatidylethanolamine (PE) was not clear. The interesting point is that the NMR signal was very strong for both PC and SM, indicating that they were present in much greater concentrations than PS and PE. This was the inverse of the results shown in Table 1 and the cause was not identified. We also found that the chemical shifts (resonance position) for individual lipids were affected by the presence of other lipids in the sample, complicating quantification of the lipid composition. Therefore, the use of NMR was not pursued further.

Cholesterol was extracted from 0.1mg of isolated membrane protein using chloroform: isopropanol:Triton X-100 (7:11:0.1 by vol). The amount of cholesterol recovered in the chloroform fraction was determined using the BioVision cholesterol assay (Mountain View, CA) according to the manufacturer's instructions. Since membranes contain cholesterol and not cholesterol esters, cholesterol esterase was not used to catalyze hydrolysis of fatty acids from cholesterol esters prior to analysis. In this assay cholesterol oxidase is used to oxidize the hydroxyl group on cholesterol to the ketone with the concomitant production of hydrogen peroxide. The hydrogen peroxide reacts with the probe and the color produced visualized using a wavelength of 570nm. Known concentrations of cholesterol were used as standards.

Gender, age, and ADI-R score are given in Table 1 for children from whom samples were used for analysis of cholesterol (all samples) and GM1 (samples marked with an asterisk). Significant differences in cholesterol content were not observed between samples isolated from RBCs from either control males or females, nor were age-related differences apparent in the results from samples obtained from control children ages 4 through 8 yrs. This was important to assess because of differences between the affected and control groups. There were significantly more girls in the comparison group and the average age was somewhat older (6.0 years for controls; 5.13 years for affected). Based on previous observations indicating cholesterol levels in blood could be altered in children with autism [5, 8] and because cholesterol is not only a component of cell membranes but is an essential component of erythrocyte lipid rafts [20], its level in membranes isolated from RBCs was determined. A significant decrease ($\rho = 0.012$, 2-tailed t-test) was seen in the average amount of cholesterol in membranes isolated from RBCs from children diagnosed with autism compared to those isolated from controls (see Figure 2A). No correlation was seen between scores obtained using the ADI-R and cholesterol levels (Figure 2B).

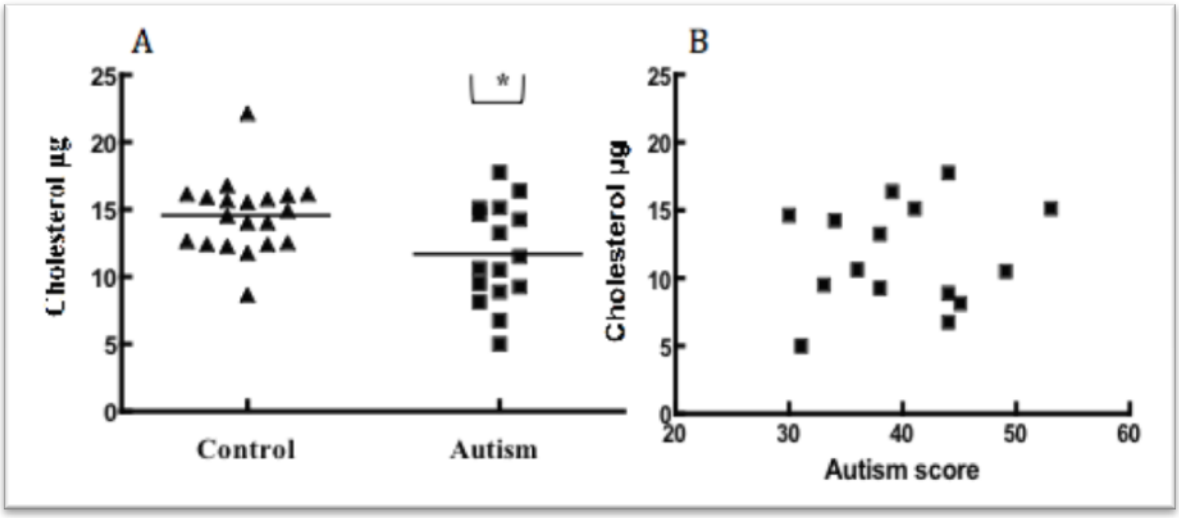

Figure 2: Cholesterol found in RBC membrane samples containing 0.1mg of protein. A) Values obtained for cholesterol present in RBC membranes isolated from blood from controls compared to those from children with autism, B) cholesterol values versus autism scores. No consistent trend between cholesterol content and autism score was noted. On average samples from children with autism had significantly less cholesterol than those from controls ($\rho<0.05>0.01$, unpaired, two-tailed student t-test).

The lack of correlation between scores obtained using the ADI-R and cholesterol levels (Figure 2B) was evident not only for the total score but for subscales that measured level of impairment of social interaction, limitation of communication and presence of repetitive and stereotypic behaviors as well. While a relationship would add to the potential significance of the current observations, the Autism Diagnostic Interview-Revised was devised to provide a clear diagnosis of classic autism for research purposes. In initial investigations like this one, it is critical to study a sample of children who clearly have autism as it is currently recognized. Because the ADI-R was not designed to quantify symptoms in the domains it samples (social atypia, communication dysfunction and repetitive, stereotypic and restrictive behaviors) in future studies it will be important to use instruments to evaluate the range of autism-associated symptoms to allow for potential correlation of behavioral phenotype with lipid profile.

While the results indicate a significant average decrease in cholesterol content of RBC membranes isolated from children with autism it can be seen that some of the values were in the normal range, in agreement with previous observations [8] indicating that only 19% of children with autism had significantly lower serum cholesterol levels than controls. Evidence that alterations in cholesterol metabolism may occur in children with autism is provided by the observation that expression of ApoB-100, a protein needed for transport of very low density lipoproteins (VLDL) from the liver to the circulation as well as for uptake of low density lipoproteins (LDL) from the circulation, was lower in children with autism compared to controls (21). Interestingly, in a case controlled study of 29 autistic boys and 29 similar male controls [22] no significant difference in the levels of total serum cholesterol and LDL-cholesterol were observed while a significant reduction in high density lipoprotein-cholesterol and a significant increase in triacylglycerides (TAGs) were found. These observations might be explained by failure of cells to appropriately metabolize TAG-rich VLDLs produced by the liver to LDLs as well as by a failure of cells to take up the LDLs that are produced. The latter may reflect altered expression of LDL receptors on the cell surface. Their finding of elevated LDL/HDL ratios in the autistic cohort supports the observation that adults with autism spectrum disorder are more apt to be diagnosed with hyperlipidemia and as a result are considered to be at risk for developing coronary heart disease [23]. While these observations might appear to contradict the hypothesis that there may be a role for cholesterol in the treatment of autism [24], that recommendation was made for autistic children found to be deficient in 7-dehydrocholesterol reductase. Overall, accumulating evidence supports the fact that a significant number of children diagnosed with autism spectrum disorder have alterations in cell cholesterol content/metabolism.

The observation that cholesterol concentration was reduced in RBC membranes obtained from children with autism led us to determine the relative amounts of GM1, a known component of RBC lipid rafts [25, 26] in membranes of RBCs isolated from both children diagnosed with autism and controls (* next to the age of children indicated in Table 1 identifies samples analyzed for GM1). *Presence of GM1* in RBC membranes was monitored using slot/dot blot technology [27,28]. Two µg of RBC membrane protein was blotted (Minifold® II Slot-Blot System, Whatman, VWR International) onto a nitrocellulose membrane according to the manufacturer's directions. Each sample was run in duplicate. Membranes were blocked for 1hr at room temperature using 5% nonfat dried milk in phosphate buffered saline prior to probing overnight at 4˚C with horse radish peroxidase conjugated binding subunit of cholera toxin (CTxB, List Biological Laboratories, Campbell, CA) in PBS containing 2% nonfat dried milk.

Bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate™ followed by exposure in a Fuji Film LAS 3000. Multigauge software was used to calculate intensities. GM1 in erythrocytes was first identified chemically by Kundu et al [29].

While the sample size was limited, the results from slot blot analyses (Figure 3) indicate that there was on the average significantly more ($\rho = 0.019$, unpaired, 2-tailed t-test) GM1 in the membranes of RBCs isolated from children with autism than controls. Analysis of the adherence of the binding subunit of cholera toxin to blots of proteins separated by SDS-PAGE indicated the presence of a single positive band that ran at the front of the blot. The observed decrease in cholesterol coupled with the increase in expression of GM1 in RBC membranes from children diagnosed with autism shown in Figure 4, is in agreement with our previous observations that loss of membrane cholesterol was accompanied by an increase in GM1 [30,31].

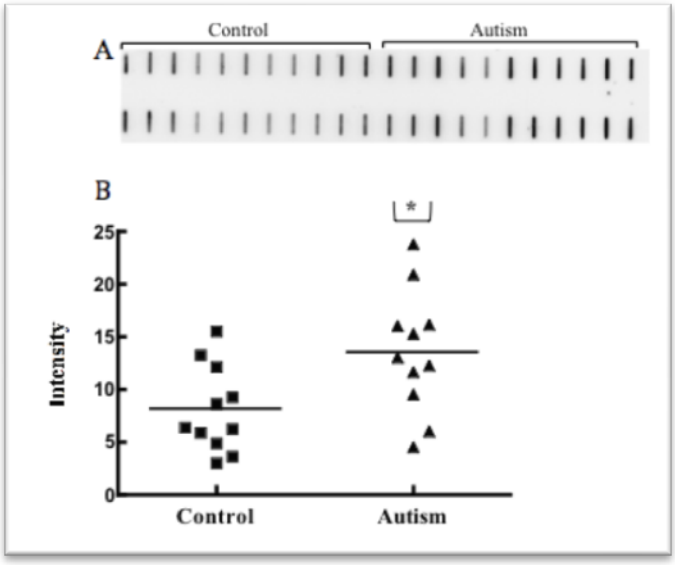

Figure 3: Relative amount of horseradish peroxidase conjugated CTxB bound to slot blots of RBC membranes isolated from the blood of children with autism relative to those from controls. A) Duplicate blots were prepared using samples of red blood cell membranes containing 2µg of protein isolated from blood obtained from eleven different children with autism and a similar number of samples of blood from controls. GM1 was detected using the HRP-conjugated binding subunit of cholera toxin and bands visualized using Super Signal West Femto Maximum Sensitivity Substrate™ and exposure using a Fuji Film LAS 3000. B) Intensity is given as the (average arbitrary units obtained for duplicates of each sample minus the arbitrary units for the background) X 10^{-5}. Samples from children with autism had significantly more GM1 than those from controls (*, $\rho<0.05>0.01$, unpaired, two-tailed student t-test).

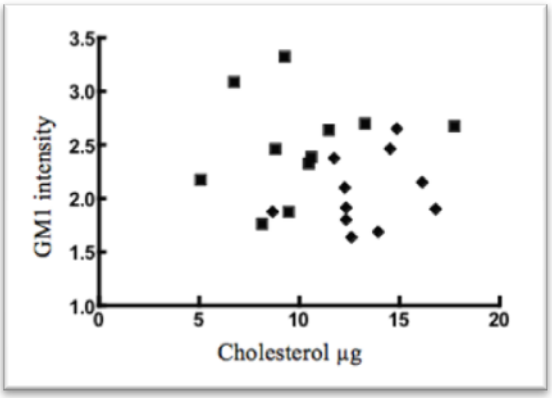

Figure 4: Plot of the intensity of CTxB binding to samples from children diagnosed with autism, squares, and controls, diamonds, versus their cholesterol concentration. Note that while many of the points for children with autism are higher, even though their cholesterol values may be lower, than those for controls.

Discussion

While changes in anisotropy were not observed for RBC membranes isolated from the blood of children with autism relative to those isolated from controls, and initial results indicated no significant differences in their phospholipid composition, the finding of differences on the average in cholesterol and GM1 support the concept that changes in lipid composition may contribute to the autistic phenotype.

Cholesterol accounts for between 25 and 50% of lipid found in plasma membranes [32] and is the source of detergent resistance of lipid rafts [33]. The latter point is substantiated by the fact that use of MβCD to acutely deplete membrane associated cholesterol results in disruption of lipid rafts [34]. We also found that exposure of N2a murine neuroblastoma cells to MβCD resulted in disruption of lipid rafts and that this disruption was accompanied by an increase in expression of the ganglioside GM1 [30], another lipid associated with rafts. Because lipid rafts are known to function in signal transduction and more specifically are necessary for normal functioning of the oxytocin receptor which can affect behavior [35], it is possible that a generalized disorder in expression of cell membrane associated cholesterol and other raft-associated lipids is associated with onset of developmental disorders identified as autism.

The significant decrease in average cholesterol content of RBC membranes isolated from blood taken from children with autism was accompanied by a similarly significant increase in GM1. Recognizing that CTxB has been reported to bind to a small protein as well as GM1 [36] it is possible that the change in GM1 seen in this study was actually a change in expression of a small peptide. While this possibility needs to be addressed, it would not negate our observation that CTxB binding was on the average enhanced in children diagnosed with autism. Because of its high affinity (found to be $4.61X10^{-12}$ using surface plasmon resonance [37]), for GM1, CTxB has been used by many [e.g. 38] as a cytochemical marker for GM1 in cells known to express it and it has been used in studies of erythrocytes [25, 26]. The presence of GM1 in human erythrocytes, shown chemically by Kundu et al [29], was reported to be the ligand recognized by CTxB when it bound to lipid rafts in erythrocyte membranes [25, 26]. The increase in CTxB binding found using slot blot analysis parallels previous observations indicating that the concentration of GM1, as well as GD1a, GD1b, and GT1b was greater in cerebral spinal fluid from autistic children than in that from controls [39, 40]. An increase in expression of GM1 could account for the reported increase in anti-GM1 autoantibodies found in serum from children diagnosed with autism [20]. The average decrease in cholesterol and increase in GM1 agrees with previous findings that a significant decrease in membrane cholesterol was accompanied by a significant increase in GM1, again identified by binding of CTxB, in neuroblastoma cells [30, 42] and in SH-SY5Y human neuroblastoma cells expressing the H63D variant of the hemochromatosis protein found in lipid rafts [31]. The fact that GM1 is associated with proteins involved in signal transduction (e.g.: cAMP kinase [43], and mitogen-activated protein kinase [44]) supports the hypothesis that these changes may be indicative of changes in signal transduction not only in RBCs but in other cells, including those in the central nervous system.

While the results presented support the concept that changes in lipid composition may contribute to the autistic phenotype, they also point out the need to ascertain whether children with autism exhibit changes in expression of proteins involved in cholesterol uptake/release by cells, the expression of enzymes involved in glycosphingolipid metabolism, and whether changes

anticipated based on the above results affect expression of proteins known to affect behavior. As more information about the expression and underlying metabolism of cholesterol and glycosphingolipids, major lipid components of membrane associated lipid rafts, by individuals affected with different neurological problems becomes known it should become possible to identify their specific effects. The observations presented point out the need for further investigation of lipid metabolism in autistic subjects the results of which could provide insights into development of new treatment strategies for autism spectrum disorders.

Presentation: Lipid raft lipid composition may affect neuronal function, CL. Schengrund, F. Ali-Rahmani, J.C. Ramer, and J.R. Connor. Talk in the colloquium "Glycobiology of the Nervous System" at the American Society for Neurochemistry 2012 meeting, Baltimore, MD. Participation in this colloquium resulted in my being asked along with Dr. R. Yu to edit a book by the same name for publication by Springer.

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39. Nordin V, Lekman A, Johansson M, Fredman P, Gillberg C (1998) Gangliosides in cerebral spinal fluid in children with autism spectrum disorders. Dev Med Child Neurol 40:587-594
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41. Mostafa GA, Al-Avadhi LY (2011) Increased serum levels of anti-ganglioside M1 auto-antibodies in autistic children: relation to disease severity. J Neuroinflammation 8:39
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18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__√___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__√___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White

_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__√__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication, listed in the table, in a PDF version 5.0.5 format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.Cholesterol, GM1, and Autism	Schengrund, Ali-Rahmani, and Ramer	Neurochem. Res.	October, 2011	☐Submitted ☐Accepted √ Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No____√_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

As indicated in the discussion for item 17, our results support the concept that changes in lipid composition may contribute to the autistic phenotype. They also point out the need for additional research to ascertain whether children with autism exhibit changes in expression of proteins involved in cholesterol uptake/release by cells, the expression of enzymes involved in glycosphingolipid metabolism, and whether changes anticipated based on the above results affect expression of proteins known to affect behavior. As more information about the expression and underlying metabolism of cholesterol and glycosphingolipids, major lipid

components of membrane associated lipid rafts, by individuals affected with different neurological problems becomes known it should become possible to identify their specific effects. The observations presented point out the need for further investigation of lipid metabolism in autistic subjects the results of which could provide insights into development of new treatment strategies for autism spectrum disorders.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____√_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____√_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

The P.I. has retired and no longer has a functioning lab.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Schengrund, Cara-Lynne	Professor, Biochemistry & Molecular Biology
eRA COMMONS USER NAME CSchengrund1	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Upsala College, East Orange, NJ	BS	1962	Chemistry
Seton Hall University, South Orange, NJ	MS	1965	Chemistry
Seton Hall University, South Orange, NJ	PhD	1966	Chemistry

A. Personal Statement:
My overall interest is in the biological role(s) of glycosphingolipids (GSLs). This has resulted in work in my lab following two distinct paths: one is to study the normal cellular function of these lipids, the other is to study their role(s) as receptors for different pathogens. It is the former work that is more akin to that described in this research.

B. Positions and Honors:

Positions

1967 Instructor, Organic Chemistry, Upsala College, East Orange, NJ.
1967–1969 Research Associate, Columbia University, College of Physicians and Surgeons, Department of Biochemistry.
1969–1972 Department of Biochemistry and Molecular Biology, Milton S. Hershey Medical Center of The Pennsylvania State University: Research Associate.
1972–1979 Assistant Professor.
1979–1992 Associate Professor.
1986–1987 Acting Department Chair.
1992–2011 Professor
2011- Professor emeritus
1995-1999 Elected Council Member of the American Society for Neurochemistry
1997–1998 NIH (sabbatical leave)
2000–2001 Chair University Faculty Senate, CIC Academic Leadership Program Fellow
Have served on a number of NIH study sections and on the VA Merit Review subcommittee for Neurobiology-D
Member ASBMB, AAAS, ACS, ISN, ASM, and the ASN

Honors

1987 Distinguished Alumni Award from Upsala College
2001 YWCA of Greater Harrisburg's 2001 Tribute to Women of Excellence

C. Peer-reviewed Publications (selected)

1. Schengrund, C-L,, F. Ali-Rahmani, and J.C. Ramer Cholesterol, GM1, and autism. Neurochem. Res. 37:1201-1207 (2012).
2. Ali-Rahmani, F., J.A. Hengst, J.R. Connor, and C-L. Schengrund Effect f HFE variants on sphingolipid expression by SH-SY5Y human neuroblastoma cells. Neurochem. Res. 36:1687-1696 (2011).
3. Schengrund, C-L. Lipid rafts: keys to neurodegeneration. Brain Res. Bull. 82: 7-17 (2010).
4. Rosa Borges, A., L. Wieczorek, B. Johnson, A.J. Benesi, B.K. Brown, R.D. Kensinger, F. L.

Krebs, B. Wigdahl, R. Blumenthal, A. Puri, F.E. McCutchan, D.L. Birx, V.R. Polonis, and C-L. Schengrund Multivalent dendrimeric compounds containing carbohydrates expressed on immune cells inhibit infection by primary isolates of HIV-1. Virology 408: 80-88 (2010).

5. Petro, K.A. and C-L. Schengrund Membrane raft disruption promotes axonogenesis in N2a neuroblastoma cells Neurochem. Res. 34:29-37 (2009).
6. Blome, M.C., B.C. Yowler, R. O'Keeffe, N. Panjwani, A.M. Pickett, and C-L. Schengrund Use of surface plasmon resonance to characterize binding of botulinum type A toxin-haemagglutinin complex to gangliosides. The Botulinum J. 1(1): 88-99 (2008).
7. Blome, M.C. and C-L. Schengrund Multivalent binding of ricin to bovine serum albumin-based neoglycoconjugates. Toxicon 51: 1214-1224 (2008).
8. Schengrund, C-L. Glycoconjugates: Roles in neural diseases caused by exogenous pathogens.CNS & Neurological Disorders–Drug Targets. 5: 381-389 (2006).
9. Hartmann, S.U., B. Wigdahl, E.B. Neely, C.M. Berlin, C-L. Schengrund, H-M Lin, and M.K. Howett Biochemical analysis of human milk treated with sodium dodecyl sulfate, an alkyl sulfate microbicide that inactivates human immunodeficiency virus type 1. J. Hum. Lact. 22(1): 61-74 (2006).
10. Urdaneta, S., B. Wigdahl, E.B. Neely, C.M. Berlin, Jr., C-L. Schengrund, H-M. Lin, and M.K. Howett Inactivation of HIV-1 in breast milk by treatment with the alkyl sulfate microbicide sodium dodecyl sulfate (SDS) Retrovirology http://www.retrovirology.com/content/2/1/28 (2005).
11. Rosa Borges, A and C-L. Schengrund Dendrimers and antivirals: a review. Current Drug Targets–Infectious Diseases. 5: 247-254 (2005).
12. Stine, R., M.V. Pishko, and C-L. Schengrund Comparison of glycosphingolipids and antibodies as receptor molecules for ricin detection. Anal. Chem. 77: 2882-2888 (2005).
13. Yowler, B.C. and C-L. Schengrund Botulinum neurotoxin A changes conformation upon binding to ganglioside GT1b. Biochemistry 43: 9725-9731 (2004).
14. Kensinger, R.D., B.C. Yowler, A.J. Benesi, and C-L. Schengrund Synthesis of Novel, Multivalent, Glycodendrimers as Ligands for HIV-1 gp 120. Bioconjugate Chemistry15: 349-358 (2004).
15. Kensinger, R.D., B.J., Catalone, F.C. Krebs,, B. Wigdahl, and C-L. Schengrund Novel Polysulfated Galactose-Derivatized Dendrimers as Binding Antagonists of HIV-1 Infection. Antimicrobial Agents and Chemotherapeutics 48: 1614-1623 (2004).
16. Schengrund, C-L. "Multivalent" Saccharides: Development of New Approaches for Inhibiting the Effects of Glycosphingolipid-Binding Pathogens. Biochem. Pharmacol. 65: 699-707 (2003).

D. Grant Support:

Completed Research Support

Collaborative Dean's Feasibility Award 2009-2011 No Number Assigned
Autism indicators: erythrocyte membrane fluidity and/or lipid composition.
The proposed studies are designed to determine whether there is a consistent change in fluidity of membranes isolated from erythrocytes from children with autism relative to that of erythrocytes from age, gender, and geographic residence matched comparison children who are developing typically.

PHS: R21 AI071877-01 (PI) 9/15/07-8/31/10
Can mannosylated dendrimers inhibit HIV-1 infection of DC-SIGN expressing cells.
The proposed studies are designed to develop multivalent mannosylated compounds that can inhibit infection of dendritic cells by HIV-1.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Jeanette C. Ramer, M.D.	Professor of Pediatrics

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Moravian College, Bethlehem, PA	B.S.	1973	Biology
PA State University Coll of Med, Hershey, PA	M.D.	1977	
PA State University Coll of Med, Hershey, PA	Genetics Fellowship	1980	

Professional Experience:

1977-1980 Pediatric Resident, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, PA
1980-1982 Instructor in Pediatrics, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, PA
1982-1992 Assistant Professor of Pediatrics, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, PA
1992-2006 Associate Professor of Pediatrics, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, PA
2006- Professor of Pediatrics, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, PA
1998- Director, Pediatric Medical Rehabilitation, Penn State Milton S. Hershey Medical Center Children's Hospital, Hershey, PA

Publications:

1. Ramer JC, Ladda RL, Frankel C: Brief Clinical Report: Two infants with del(3)(p25pter) and a review of previously reported cases. Am J Med Gen 33:108-112, 1989.
2. Ramer JC, Ladda RL: Brachydactyly, sensorineural hearing loss, skeletal anomalies and mental retardation in a male. Dysmorphol Clin Genet 3(2), 1989.
3. Ramer JC, Ladda RL: Report of a male with feature overlapping Geroderma Osteodysplastica and Mandibuloacral Dysplasia. Dysmorphol Clin Genet 4(2):66-78, 1990.
4. Ramer JC, Mowrey PN, Robins DB, Ligato S, Towfighi J, Ladda RL: Five children with del (2) (q31q33) and one individual with dup (2)(q31a33) from a single family: Review of brain, cardiac and limb malformations. Am J Genet 37:392-400, 1990.
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C. Research Support

Ongoing Research Support:

1.*"Assessment and treatment of copper/zinc imbalance in children with autism"*
Funded 7-1-06 through 12-31-10 Thrasher Research Fund, Salt Lake City UT 84101
Dr. Ramer is PI for this project. This study evaluates copper and zinc levels, as well as copper to zinc ratio, in children with autism (ages 3-8 years) and compares them to control children. The second phase, which is just beginning, will provide oral supplementation (using a double blind, placebo controlled protocol) with zinc and vitamin C for those children with autism whose Cu/Zn is above a value that is calculated to be significantly different from the mean value for typically developing children.

2."*A Phase III Randomized, Double-blind, Placebo-controlled Study to Evaulate the Safety and Efficacy of Drug A in children with Autism"*
Funded 9/19/2009-9/18/2011 Curemark, LLC 206 Theodore Fremd Avenue, Rye, NY 10580
Total budget $142,406
Dr. Ramer is PI for this site which is part of a national consortium.