AWARD NUMBER: W81XWH-17-1-0060

TITLE: Functional genetics for predisposition to development of Type 2 Diabetes in obese individuals

PRINCIPAL INVESTIGATOR: Assia Shisheva

CONTRACTING ORGANIZATION: Wayne State University, Department of Physiology

REPORT DATE: OCTOBER 2020

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2020	Final	15APR2017 - 14JUN2020

4. TITLE AND SUBTITLE
Functional genetics for predisposition to development of Type2 Diabetes in obese individuals

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0060

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Assia Shisheva, Berhane Seyoum, Diego Sbrissa, Ognian Ikonomov

E-Mail: ashishev@med.wayne.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wayne State University
Department of Physiology
4237 Scott Hall,
540 E. Canfield
Detroit, MI 48201-1928

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Obesity is a major risk factor for Type 2 Diabetes (T2D) yet the majority of obese subjects never develop T2D and their metabolic tissues never lose insulin sensitivity. This suggests the key role of genetic predisposition to T2D development in obesity. This proposal assesses for the first time whether single nucleotide variations (SNVs) within three genes, *PIKFYVE*, *FIG4* and *VAC14*, encoding proteins that assemble in a single complex to regulate glucose homeostasis, are associated with T2D in obesity. We performed exome sequencing in abdominal fat derived from bariaric surgeries of 96 morbidly obese individuals with of without T2D (48 subjects/group). The two groups were with similar average BMI and age. We identified total of seven SNVs in the three genes (2 in *PIKFYVE*, 3 in *VAC14* and 2 in *FIG4*) associated with T2D ($p<0.05$; n=48) by logistic regression analysis. The T2D-asssociated SNVs were all located in the noncoding regions of the three genes, had already assigned id numbers and were still not linked to a disease. Intriguingly, several of the candidate T2D-associated SNVs were markedly more frequent in the group of the morbidly obese women (n=32). Analysis for SNVs associated with T2D in women's group uncovered two additional variants (id 2118296; id10208655) located in the adipose super-enhancer of the *PIKFYVE* gene. A constellation of these two SNVs and one of the identified *PIKFYVE* SNV (id 6755550) was seen in 25% of the morbidly obese women with T2D but not in those without T2D (n=32). Attempts were also made to relate the T2D-associated SNVs to the PIKfyve enzyme activity, measured *in vitro* in fat tissue. Together, our results support the hypothesis that single nucleotide variations in the three genes of interest are associated with T2D in morbidly obese patients. Our observation that some T2D-associated SNVs are located within the adipose super-enhancer of the *PIKFYVE* gene is consistent with the notion that these SNVs might affect PIKfyve expression in fat tissue in T2D.

15. SUBJECT TERMS
type 2 diabetes mellitus; morbid obesity; *PIKFYVE*, *FIG4* and *VAC14* genes; single nucleotide variations;

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION: Patients suffering from Type 2 Diabetes mellitus (T2D) are usually overweight or obese. However, only a small fraction of even morbidly obese individuals develops T2D. Since T2D is a familial disease, a genetic predisposition for the development of T2D is expected to play a major role in obese individuals. Basic studies have shown that manipulating the levels of 3 cellular proteins, i.e., PIKfyve (encoded by *PIKFYVE* gene), Sac3 (encoded by *FIG4* gene) and ArPIKfyve (encoded by *VAC14* gene) – which assemble in a functional complex, markedly affects insulin sensitivity of fat cells [1, 2]. Furthermore, knockout of mouse *pikfyve* selectively in muscle or fat cells causes whole body insulin resistance in transgenic mice [3, 4]. Therefore, it is plausible that mutations in the three genes of interest could cause tissue insulin resistance and thereby could predispose the obese individuals to develop T2D.

2. KEYWORDS: T2D (type 2 diabetes mellitus); morbid obesity; *PIKFYVE*, *VAC14* and *FIG4* genes; SNV (single nucleotide variations)

3. ACCOMPLISHMENTS:

Identifying SNVs in the coding or noncoding regions in PIKFYVE, VAC14 and FIG4 genes and their association with T2D in morbidly obese patients

As stated in our previous reports and indicated below for readers' convenience, our cohort of 96 patients comprised 48 morbidly obese individuals without type 2 diabetes (T2D) and 48 morbidly obese individuals with T2D, as shown in Table 1. Age and BMI were not statistically different between the two groups.

Table 1. Groups, Age and BMI

	Morbidly obese without T2D	Morbidly with T2D
Total	48	48
Age (mean $\pm$ SEM)	42.0 $\pm$ 2.85	47.0 $\pm$ 1.5
BMI (mean $\pm$ SEM)	49.4 $\pm$ 1.06	47.9 $\pm$ 1.01

Genomic DNA was extracted from small samples of abdominal fat excised during bariatric surgery of the 96 morbidly obese patients. As also noted in our last annual report, we had to repeat 5 genomic DNA samples due technical failure. These samples, together with a positive control, were successfully sequenced and the data included in our final analysis**.** Exon sequencing combined with genome database searches for the 3 genes of interest- *PIKFYVE*, *VAC14* and *FIG4* - identified 942 single nucleotide variations (SNVs) in the total of 96 genomic DNA samples (Table 2). As could be expected, the number of SNVs was proportional to the number of exons in each gene of interest (Table 2).

Table 2. General characteristics of the 3 genes of interest, encoded protein sizes, and number of detected SNVs

Gene	Chromosome	Gene size (kB)	Exons	Protein (kD)	SNVS
PIKFYVE	2q34	99.5	42	237	434

VAC14	16q22.1-q22.2	120.7	19	88	216
FIG4	6q21	141.2	23	102	292
Total					942

Interestingly, only 4 SNVs were found in the exons (coding region) of *PIKFYVE* and *FIG4;* one synonymous and one non-synonymous in each of the two gene. However, none of those four SNVs showed statistically significant association with T2D as identified by logistic regression analysis (not shown).

We identified seven intronic (non-coding) SNVs associated with T2D ($P<0.05$) using logistic regression analysis of morbidly obese without T2D (n=48) vs. morbidly obese individuals with T2D (n=48): 2 in *PIKFYVE*, 3 in *VAC14* and 2 in *FIG4* (Table 3). As discussed further, the statistical significance of the T2D association for certain SNVs was much grater in the females than in males (Table 3). All candidate SNVs have already established reference identification numbers in genomic databases (Table 3), however, without reported association with pathological conditions.

Table 3. SNVs in the 3 genes associated with T2D in morbidly obese individuals

	Gene	SNV (nucleotide)	Rs (Id#)	Total (n = 96)	Women (n = 64)	Men (n = 32)
1	*PIKFYVE*	208300763 A>T	6755550	P=0.02	P=0.0001	P=0.18
2	*PIKFYVE*	208312083 T>G	12624141	P=0.04	P=0.003	P=1.0
3	*VAC14*	70782990 C>T	1875941	P=0.03	P=0.0002	P=0.22
4	*VAC14*	70697399 G>A	2242126	P=0.04	P=0.06	P=0.04
5	*VAC14*	70698580 G>A	7201952	P=0.05	P=0.11	P=0.43
6	*FIG4*	109766412 T>C	17612996	P=0.05	P=0.11	P=0.33
7	*FIG4*	109785994 G>A	9386844	P=0.03	P=0.18	P=0.1
8	*PIKFYVE*	208347642 G>A	2118296	P=0.2	P=0.005	P=0.22
9	*PIKFYVE*	208342938 A>G	10208655	P=0.22	P=0.0003	P=0.023

For comparison, we analyzed 6 other genes, i.e., *PNPLA3*; *TM6SF2*; *MBOAT7*; and the 3 members of CALPONIN gene family (*CNN1*, *-2* and *-3*), all of which are functionally unrelated to our three genes of interest (*PIKFYVE*, *VAC14* and *FIG4*). Combined, these genes contain 46 exons, a number comparable to the 42 exons in *PIKFYVE* or the 42 exons in *VAC14* plus *FIG4* combined (Table 4).

Table 4. "Control" genes: chromosome location, size, exons and encoded proteins

	Gene	Chromosome	Gene size (kB)	Exons	Protein (mass kD)
1	*PNPLA3*	22q13.31	40.8	9	52.9
2	*TM6SF2*	19p13.3-p12	19.3	10	42.5
3	*MBOAT7*	19q13.42	16.5	6	52.7
4	*CNN1*	19p13.2-13.1	11.7	7	33.2
5	*CNN2*	19p3	12.5	7	33.7
6	*CNN3*	1p22-p21	30.1	7	36.4
	Total			46	251.4

We detected a total of 412 SNVs in the 6 "control" genes. Intriguingly, in comparison with to the 2 SNVs in *PIKFYVE* and the 5 SNVs in *VAC14* plus *FIG4* (Table 3) there was just one intronic SNV in the 6 genes together, found to be associated with T2D - in *PNPLA3*; chr. 22 43946106; id 2294917. Together, these data suggest that the SNVs in *PIKFYVE*, *VAC14* and *FIG4* genes, indicated in Table 3, found associated with T2D in morbidly obese individuals may be involved in the predisposition of the T2D in obesity. Future studies are needed to identify their precise relationship with T2D.

Several T2D-associated PIKfyve or VAC14 SNVs in morbidly obese individuals are more frequent in women than in men

Of the nine T2D-associated SNVs, illustrated in Table 3, # 1, 2, 3, 8 and 9 were markedly more frequent in women with T2D than in men with T2D. The other 2 *VAC14*

candidate SNVs and the two *FIG4* SNVs did not exhibit pronounced sex-dependent distribution (Table 3).

The 2x3 contingency tables for all patients, and separately for women and men of the *PIKFYVE* 208300763 A>T variant are illustrated in Table 5. The analyses indicate that the statistical significance of the *PIKFYVE* 208300763 variant association with T2D based on the two groups (each with 48 individuals) is due to the much higher degree of association in the group of females than that of males. Thus, the Freeman-Halton extension of the Fisher exact probability test [5] determined significant association with T2D with P=0.024 for all individuals (n = 96) but much higher for women (P=0.00012; n=64; each group with 32 individuals). The P value for the T2D and non-T2D man groups (n=32; each group with 16 individuals) was 0.19 (not significant).

Table 5. Contingency analysis of the *PIKFYVE* 208300763 A>T variant shows greater association with T2D in females than in males

208300763	TT*	TA^	AA#
Morbidly obese No T2D (n=48)	18	0	30
Morbidly obese + T2D (n=48)	29	1	18
Women No T2D (n=32)	3	0	29
Women + T2D (n=32)	17	1	14
Men No T2D (n=16)	15	0	1
Men + T2D (n=16)	12	0	4

*, homozygous mutation; ^, heterozygous mutation; #homozygous reference

Likewise, analyses of the 2x3 contingency tables for *PIKFYVE* 208347642 G>A also illustrates that this variant is more frequent in women than in men as illustrated in Table 6 (for P values, see Table 3).

Table 6. Contingency analysis of the *PIKFYVE* 208347642 G>A variant showing association with T2D in women

208347642	AA*	AG^	GG#
Women No T2D (n=32)	2	0	30
Women + T2D (n=32)	12	0	20
Men No T2D (n=16)	14	0	2
Men + T2D (n=16)	10	1	5

*, homozygous mutation; ^, heterozygous mutation; #homozygous reference

Similarly, analysis of the 2x3 contingency tables for *PIKFYVE* 208312083 T>G showed that this variant is more frequent in women than in men (for P values, see Table 3).

A similar pattern of predominant association with T2D in women was also observed for the *VAC14* association candidate SNV 70782990. In this case SNV 70782990_T was detected in 4 DNA samples from morbidly obese women without T2D and 19 morbidly obese women with T2D (Table 7) (for P values, see Table 3).

Table 7. Contingency analysis of the *Vac14* 70782990 C>T variant shows greater association with T2D in women than in man

70782990	TT*	TC^	CC#
Morbidly obese No T2D	17	3	28

(n=48)			
Morbidly obese + T2D (n=48)	27	6	15

Women No T2D (n=32)	4	0	28
Women + T2D (n=32)	18	1	13

Men No T2D (n=16)	13	3	0
Men + T2D (n=16)	9	5	2

*, homozygous mutation; ^, heterozygous mutation; #homozygous reference

The in vitro PIKfyve activity not different between the T2D and non-T2D morbidly obese subjects

We first sought to identify if the presence of T2D will alter PIKfyve activity in the morbidly obese patients. To address this question, we measured the PIKfyve activity by an in vitro assay that we described previously [6]. The activity was quantified from the amount of PtdIns5P and PtdIns(3,5)P2 synthesized by PIKfyve preparations immunopurified from lysates of the patients' abdominal fat. PIKfyve immunoprecipitates immobilized on protein A-Sepharose were incubated in presence of PtdIns as a substrate and labeled [^{35}P]-gamma ATP [6]. The PIKfyve activity for given individual was quantified by normalizing for the amount of the lipid products generated by a PIKfyve standard and presented as percentage. It should be noted that the assay requires significantly greater amounts of abdominal tissue than that available at our disposal; therefore only ~50% of our cohort underwent the activity assay.

Our results indicated that the two PIKfyve products PtdIns(3,5)P2 and PtdIns5P showed very high correlation (r = 0.8) in all successfully processed samples (n= 47). This is

consistent with the notion that the produced two lipids are dependent on the immunoprecipitated amount of PIKfyve protein.

However, the data for the PIKfyve activity in diabetic vs. non-diabetic groups did not show statistically significant differences. Whereas this result could be consistent with a lack of PIKfyve activity alterations in T2D, the inherent limitations of the study such as data variability and the small number of participants per group, may mask potential T2D-related changes in the PIKfyve activity.

Effect of the T2D-associated SNVs on the in vitro PIKfyve activity

The protein products of the three genes, i.e., *PIKFYVE*, *VAC14* and *FIG4* are PIKfyve, ArPIKfyve and Sac3, respectively. Previous studies have indicated that the three protein interact in a single protein complex (called the PAS complex) that controls both synthesis and turnover of PtdIns5P and PtdIns(3,5)P2 [7]. As mentioned above, the in vitro measured PIKfyve activity remained unaltered as a function of T2D, therefore we next sought to identify a potential relationship between the candidate SNVs in the three genes and the PIKfyve enzymatic activity.

We analyzed a potential relationship between the two independent factors under study - the candidate SNVs and T2D - with PIKfyve enzymatic activity by 2-way analysis of variance (ANOVA). It should be noted that each of the three proteins, i.e., PIKfyve, ArPIKfyve or Sac3 is required for proper PIKfyve activity [8, 9].

Noteworthy, out of the identified candidate SNVs associated with T2D (Table 3), the 70782990 C>T variant in the *VAC14* gene in women was the only candidate SNV allowing 2-way ANOVA analysis with respect to the in vitro PIKfyve activity (Table 8). For the other

T2D-associated SNVs, there were just 1- 2 values or no data for the *in vitro* PIKfyve activity in some of the subgroups, thus preventing such analysis.

The two-way ANOVA analysis in females (*in vitro* PIKfyve activity under the 70782990 C>T SNV presence/absence; T2D absence/presence) shows that the row x column differences are statistically significant F=5.83; P=0.023 (Table 8). Thus, two-way ANOVA with *VAC14* 70782990 C>T suggests that the association of the variant T with T2D correlates with lower in vitro PIKfyve activity in morbidly obese women. The data with *VAC14* 70782990 C>T SNV supports the hypothesis that at least in women, the association with T2D correlates with lower PIKfyve activity, and hence, lower PIKfyve levels and consequently decreased PAS complex formation and functionality.

Table 8. Two-way Anova analysis showing that candidate T2D-associated *VAC14* 70782990 C>T SNV in women with T2D correlates with reduced PIKfyve activity

70782990	TT*	CC#
Morbidly obese No T2D	18.50 +/- 17.3 (n=3)	1.73 +/-1.18 (n=15)
Morbidly obese + T2D	1.53 +/- 0.54 (n=5)	2.20 +/- 0.88 (n=6)

*, homozygous mutation; #homozygous reference

ANOVA Summary					
Source	**SS**	**df**	**MS**	**F**	**P**
Rows	47.16	1	47.16	0.56	0.4612
Columns	209.32	1	209.32	2.48	0.1279
r x c	491.88	1	491.88	5.83	0.0234
Error	2110.39	25	84.42		
Total	2858.75	28			

Although we did not have sufficient data to determine the impact of the *PIKFYVE* SNV 208300763 A>T on the in vitro PIKfyve kinase activity, it is interesting to point out that the *VAC14* 70782990 SNV coincided with *PIKFYVE* 208300763 SNV in the samples of 12

women with T2D. By contrast, the coincident presence of the 2 SNVs was found only in one morbidly obese woman without T2D.

How the intronic SNVs in PIKFYVE and VAC14 may affect PIKfyve protein functionality in adipose tissue

It is currently accepted that SNVs located in a super-enhancer region might alter tissue-specific gene expression [10]. Super-enhancers are intronic regions outside the gene promoter, which are critical for tissue-specific protein expression as they harbor multiple binding sites for tissue-specific transcription factors. There is a single adipose-specific super-enhancer region in the *PIKFYVE* gene – from nt. 208336466 to nt. 208427375 [11]. Whereas the *PIKFYVE* SNV 208300763 A>T (Table 3) is away from the super-enhancer region, the *PIKFYVE* SNVs 208347642 G>A and 208342938 A>G (Table 3) are within the region. Interestingly, 8 of the 19 women with T2D having the SNV 208300763 also had both the 208347642 and 208342938 SNVs in *PIKFYVE* super enhancer. In contrast, this triad of SNVs was never found in the group of morbidly obese women without T2D. The combined association of 3 SNVs in *PIKFYVE* with T2D supports the notion that altered PIKfyve expression levels in the adipose tissue may play a role in the association with T2D in morbidly obese women. However, 8 out of 32 women represent just 25% of the 32 morbidly obese women with T2D. Further studies with larger number of participants are needed to test the validity of our observations.

Whereas *FIG4* does not harbor a super-enhancer sequence, *VAC14* contains a single super enhancer in liver and brain but not in adipose tissue. The *VAC14* T2D-associated SNV candidate 70782990 is located close to the super enhancer: chr. 16, from 70714716 to 70782498 [10, 11]. Therefore, if this *VAC14* variant affects expression of the ArPIKfyve protein in liver and/or brain it could indirectly affect adipose ArPIKfyve expression and, consequently, PAS complex levels.

Conclusions

Despite several limitations, such as (i) a small number of individuals in the study; (ii) limited genomic sequence for analysis (only exons and adjacent areas) and (iii) dependence of the functional variable, i.e., the *in vitro* PIKfyve activity on both obesity and/or T2D, the results of our study provide novel observations and allow several important conclusions as indicated below:

- No variations in the coding sequences of the 3 genes of interest associated with T2D in the morbidly obese patients.

This observation is somewhat unexpected as disease-causing mutations in each of the three genes are identified in other disorders. Thus, *PIKFYVE* mutations in one allele, resulting in a premature stop codon and truncated protein, cause a benign fleck corneal dystrophy [12]. Mutations in *VAC14* homo-dimerizing domain are associated with rare pediatric onset striatal neurodegeneration [13]. Compound heterozygosity for *FIG4* (one null allele and one Sac3/FIG4 ^{41}I/T substitution) causes CMT4J peripheral neuropathy [14]. In contrast to these diseases, our data suggest that the association of *PIKFYVE*, *VAC14* and *FIG4* with T2D in morbid obesity might involve alterations in the non-coding regions of the genes.

- Sex dependence of candidate SNVs and in vitro PIKfyve activity.

Although evidence for sex dependence of genomic associations with lipid metabolism and T2D has been accumulating for some time [15], here we report for the first time that certain single nucleotide variants in *PIKFYVE* and *VAC14* introns are significantly associated with T2D in women but not in men.

- Coincidence of the intronic SNVs.

We report constilation of three intronic *PIKFYVE* SNVs coinciding in 25% of the morbidly obese women with T2D but not in the morbidly obese women without T2D. Two of these SNVs are located in the adipose super-enhancer region of *PIKFYVE*. These findings should be taken into consideration in future genomics and functional studies on the role of PIKfyve complex in T2D pathogenesis.

References

1. Ikonomov OC, Sbrissa D, Dondapati R, Shisheva A: **ArPIKfyve-PIKfyve interaction and role in insulin-regulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes.** *Exp Cell Res* 2007, **313:**2404-2416.
2. Ikonomov OC, Sbrissa D, Ijuin T, Takenawa T, Shisheva A: **Sac3 is an insulin-regulated phosphatidylinositol 3,5-bisphosphate phosphatase: gain in insulin responsiveness through Sac3 down-regulation in adipocytes.** *J Biol Chem* 2009, **284:**23961-23971.
3. Ikonomov OC, Sbrissa D, Delvecchio K, Feng HZ, Cartee GD, Jin JP, Shisheva A: **Muscle-specific Pikfyve gene disruption causes glucose intolerance, insulin resistance, adiposity, and hyperinsulinemia but not muscle fiber-type switching.** *Am J Physiol Endocrinol Metab* 2013, **305:**E119-131.
4. Ikonomov OC, Sbrissa D, Delvecchio K, J AR, Shisheva A: **Unexpected severe consequences of Pikfyve deletion by aP2- or Aq-promoter-driven Cre expression for glucose homeostasis and mammary gland development.** *Physiol Rep* 2016, **4**.
5. Freeman GH, Halton JH: **Note on exact treatment of contingency, goodness of fit and other problems of significance.** *Biometrika* 1951, **38:**141-149.
6. Sbrissa D, Ikonomov OC, Filios C, Delvecchio K, Shisheva A: **Functional dissociation between PIKfyve-synthesized PtdIns5P and PtdIns(3,5)P2 by means of the PIKfyve inhibitor YM201636.** *Am J Physiol Cell Physiol* 2012, **303:**C436-446.
7. Sbrissa D, Ikonomov OC, Fu Z, Ijuin T, Gruenberg J, Takenawa T, Shisheva A: **Core protein machinery for mammalian phosphatidylinositol 3,5-bisphosphate synthesis and turnover that regulates the progression of endosomal transport. Novel Sac phosphatase joins the ArPIKfyve-PIKfyve complex.** *J Biol Chem* 2007, **282:**23878-23891.
8. Sbrissa D, Ikonomov OC, Fenner H, Shisheva A: **ArPIKfyve homomeric and heteromeric interactions scaffold PIKfyve and Sac3 in a complex to promote PIKfyve activity and functionality.** *J Mol Biol* 2008, **384:**766-779.
9. Ikonomov OC, Sbrissa D, Fenner H, Shisheva A: **PIKfyve-ArPIKfyve-Sac3 core complex: contact sites and their consequence for Sac3 phosphatase activity and endocytic membrane homeostasis.** *J Biol Chem* 2009, **284:**35794-35806.
10. Hnisz D, Abraham BJ, Lee TI, Lau A, Saint-Andre V, Sigova AA, Hoke HA, Young RA: **Super-enhancers in the control of cell identity and disease.** *Cell* 2013, **155:**934-947.
11. Sun W, Yao S, Tang J, Liu S, Chen J, Deng D, Zeng C: **Integrative analysis of super enhancers SNPs for type 2 diabetes.** *PloS one* 2018.
12. Li S, Tiab L, Jiao X, Munier FL, Zografos L, Frueh BE, Sergeev Y, Smith J, Rubin B, Meallet MA, et al: **Mutations in PIP5K3 are associated with Francois-Neetens mouchetee fleck corneal dystrophy.** *American journal of human genetics* 2005, **77:**54-63.
13. Lenk GM, Szymanska K, Debska-Vielhaber G, Rydzanicz M, Walczak A, Bekiesinska-Figatowska M, Vielhaber S, Hallmann K, Stawinski P, Buehring S, et al: **Biallelic Mutations of VAC14 in Pediatric-Onset Neurological Disease.** *American journal of human genetics* 2016, **99:**188-194.
14. Chow CY, Zhang Y, Dowling JJ, Jin N, Adamska M, Shiga K, Szigeti K, Shy ME, Li J, Zhang X, et al: **Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J.** *Nature* 2007, **448:**68-72.
15. Link JC, Reue K: **Genetic Basis for Sex Differences in Obesity and Lipid Metabolism.** *Annual review of nutrition* 2017, **37:**225-245.

4. IMPACT: Our final analysis supports the hypothesis that single nucleotide variations in the genes of interest are associated with T2D in morbidly obese patients. At this point it is

early to determine the impact on other disciplines, on technology transfer and on society. However, the observations for sex dependence of certain T2D-accosiated SNVs and the identification of a constellation of 3 SNVs in *PIKFYVE* (with 2 of them in the super-enhancer region) in 25% of morbidly obese women with T2D but not in the women without T2D suggest the SNVs involvement in the regulation of adipose PIKfyve expression/activity. The possibility that changes in PIKfyve expression in fat tissue could be causative for T2D development in a fraction of morbidly obese women is supported by adipose-specific PIKFYVE knock out in mice, which exhibit systemic glucose intolerance and insulin resistence [4]

5. CHANGES/PROBLEMS: We received a second co-cost extension due to the necessity to resequence 5 of our 96 smaples.

6. **PRODUCTS**: For the period of the award we published 4 papers, supported partly by DoD, to further characterize the pathology of PIKfyve dysfunction. We were able to finish more work because mutagenesis was not a necessary step, given that all of the SNVs associated with T2D were located in the non-coding region of the 3 genes.

Ikonomov, OC, Altankov, G, Sbrissa, D, **Shisheva, A**: PIKfyve inhibitor cytotoxicity requires AKT suppression and excessive cytoplasmic vacuolation. Toxicol Appl Pharmacol. 2018 Oct 1;356:151-158. doi: 10.1016/j.taap.2018.08.001. Epub 2018 Aug 9.

Sbrissa, D, Naisan, G, Ikonomov, OC, **Shisheva, A:** Apilimod, a candidate anticancer therapeutic, arrests not only PtdIns(3,5)P2 but also PtdIns5P synthesis by PIKfyve and induces bafilomycin A1-reversible aberrant endomembrane dilation. PLoS One. 2018 Sep 21;13(9):e0204532. doi: 10.1371/journal.pone.0204532. eCollection 2018.

Ikonomov, OC, Sbrissa, D, **Shisheva, A.** Small molecule PIKfyve inhibitors as cancer therapeutics: translational promises and limitations. Toxicol Appl Pharmacol 2019 Nov 15;383:114771.
doi: 10.1016/j.taap.2019.114771. Epub 2019 Oct 16.

Shisheva, A, Sbrissa, D, Hu, B, Li, J. Severe consequences of Sac3/Fig4 phosphatase deficiency to phoshoinositides in patients with Charcot-Marie-Tooth disease type-4J. Mol Neurobiol 2019 Dec;56(12):8656-8667. doi: 10.1007/s12035-019-01693-8. Epub 2019 Jul 16.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Note, during our 1-year no-cost extension, only the principal investigator will work on this grant under 0.6% effort.

Name, ID	Assia Shisheva
Role	Principal Investigator
Contributions	Managing all activities
Nearest Month worked	0.6
Funding support	none

8. SPECIAL REPORTING REQUIREMENTS: Note, during our no-cost extension period, only the principal investigator will work on this grant under 0.6% effort.

9. **APPENDICES:** None