Elevated Levels of miR-122 in Serum May Contribute to Improved Endothelial Function and Lower Oncologic Risk Following Bariatric Surgery

Arnon Blum MD[1,2], Hila Yehuda MSc[3], Nissim Geron MD[1] and Ari Meerson PhD[3]

[1]Department of Medicine, Padeh Poria Medical Center, Tiberias, Israel
[2]Faculty of Medicine in the Galilee, Bar Ilan University, Safed, Israel
[3]Molecular Biology of Chronic Diseases Laboratory, MIGAL Galilee Research Institute, Kiryat Shmona, Israel

ABSTRACT: **Background:** Weight loss surgery is the most effective treatment for obesity, and it reduces cardiovascular and cancer risk through poorly understood mechanisms. MicroRNAs (miRNAs) are short RNA molecules that regulate the stability and translation of many mRNAs. We hypothesized that levels of specific circulating miRNAs are altered following surgery and may contribute to lower cancer risk.

Objectives: To investigate the change of miRNA following surgery.

Methods: All patients underwent gastric "sleeve operation." RNA was isolated from sera of 21 patients (14 men, 7 women) before and 3 months after surgery. Sera were combined into two pools, which served for cDNA library construction followed by miSeq sequencing. The levels of candidate miRNAs were validated in the individual samples by QRT-PCR.

Results: Serum miR-122 was significantly up-regulated 3 months post-bariatric surgery in sera of patients, whose endothelial function had greatly improved. In addition, serum miR-122 levels correlated positively with endothelial function as measured by FMD. The changes in miR-122 levels from pre-surgery to 3 months post-surgery also tended to correlate with the respective changes in FMD.

Conclusions: The serum miR-122/miR-451 ratio may serve as a marker for endothelial function in obese patients. miR-122 is the dominant miRNA in the liver and a known tumor suppressor. Our findings suggest a role for circulating miR-122 in the maintenance of vascular endothelial cells (VECs) and in the prevention of cancer. Further studies are required to elucidate the mechanism of its secretion into circulation and its absorption by VECs, as well as its relevant cellular targets.

IMAJ 2017; 19: 620–624

KEY WORDS: obesity, cancer prevention, miRNA 122, bariatric surgery, inflammation

Obesity and obesity-associated diseases are leading health problems in developed countries. In fact, a positive association has been found between obesity and the risk for developing type 2 diabetes mellitus (T2DM), cardiovascular diseases, and multiple cancers including breast cancer and colorectal cancer [1].

Weight loss surgery is the most effective treatment for morbid obesity, and as a result, T2DM. Epidemiological studies have shown a dramatic reduction in relative risk to develop cancer in obese patients who underwent bariatric surgery, relative risk (RR) = 0.55, and the effect on cancer risk was modified by gender. Women had a greater benefit from the surgery [RR = 0.68] while in men it was less significant [RR = 0.99] [2]. Long term outcomes for surgically induced weight loss have demonstrated reduced incidence of diabetes, cardiovascular events, and cancer [3,4]. Three months following bariatric surgery, the most dramatic effects are weight loss, improvement in T2DM management, lower arterial hypertension, and a significant improvement in endothelial function [5,6]. These short-term beneficial effects have recently been shown to be linked to the inhibition of inflammation [7]. In agreement with this finding, prior studies have demonstrated a significant inhibition of C-reactive protein (CRP) and tumor necrosis factor alpha (TNF-α) 3 months post-surgery [5,6] and suggested that the reduction of inflammation and oxidative stress following the surgery can improve the function of vascular endothelial cells (VECs). Nevertheless, the mechanism of improvement in vascular endothelial function, and the reduction of cancer risk following bariatric surgery are poorly understood.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs (ncRNAs), which play regulatory roles by predominantly binding to cis-elements in the 3' untranslated region of specific mRNAs and regulating their translation or stability [7-9]. Recent studies have begun to elucidate the role of miRNAs in various biological processes including adipocyte differentiation, metabolic integration, insulin resistance, and appetite regulation [10-12], as well as deregulation of many miRNAs in metabolic tissues of obese animals and humans [11-13]. Numerous

recent studies have shown that miRNAs and other ncRNAs can be remarkably stable in biofluids, such as plasma/serum, urine, cerebrospinal fluid, and breast milk [14]. This stability appears to be conferred by the binding of proteins, or encapsulation in lipid vesicles, of the RNA molecules. Furthermore, the levels of specific ncRNAs in biofluids appear to be subject to tight regulation [15], suggesting that they may function as a novel endocrine signaling entity that are carried by the biofluid and internalized and affected by target tissues and cells far removed from the ones that produced them. For example, miR-122, the dominant miRNA in the liver [16] and a known tumor suppressor [17], has been shown to be transferred between cells [18].

To further explore the mechanisms modulated by the surgery, we checked whether the levels of specific serum miRNAs are persistently altered following the surgery, as absorption of these molecules by VECs can affect endothelial function and may have an effect on the clinical outcome.

PATIENTS AND METHODS

RNA was isolated from sera of 21 patients both before and 3 months after bariatric surgery. For initial global profiling of miRNAs, the RNA samples before and after surgery from 10 patients who showed the highest increases in flow medicated diameter (FMD) were combined into two pools, which served for cDNA library construction followed by miSeq sequencing. The levels of candidate miRNAs were validated in the individual samples by quantitative reverse transcription-polymerase chain reaction (QRT-PCR).

COHORT DESCRIPTION

The sera and FMD values were from a previously described cohort of patients undergoing gastric sleeve operations for weight loss [11]. The specific subset of the cohort consisted of 14 men and 7 women, of which samples from 7 men and 3 women were pooled for the initial RNA sequencing (RNA-Seq) profiling. The distributions of miR-122 levels and FMD values did not vary significantly between men and women in this set.

ENDOTHELIAL FUNCTION ASSESSMENT

FMD was assessed by measuring the change in the brachial artery diameter by high-resolution ultrasound. After an artery above the antecubital fossa is imaged, a blood pressure cuff placed below the antecubital fossa is inflated to suprasystolic pressure. After cuff release, reactive hyperemia is quantified. The percent change from the baseline measurement is the FMD.

RNA ISOLATION

Isolation of total RNA (including miRNAs) was carried out using the miRNeasy serum/plasma kit (Qiagen, Netherlands) according to manufacturer's instructions.

MIRNA PROFILING BY RNA-SEQ

cDNA libraries were constructed from the two sample pools using a NEBNext® Small RNA Library Prep Set for Illumina® from New England Biolabs (USA) and next-generation sequencing performed on an Illumina miSeq at the Hebrew University Genomic Center, Givat Ram, Jerusalem, Israel. Sequencing data analysis was performed using CLC Genomics Workbench software from CLCBio, version 7 (Qiagen, Netherlands).

RT-QPCR

Reverse transcription, primer design, and quantitative PCR were performed using SYBR Green chemistry dye and DNA primers. The primer extension was used for miRNA quantification as previously described [19]. This quantitative reverse transcription PCR (RT-qPCR) method is highly accurate and reproducible [19] and was chosen over hydrolysis probe chemistry due to lower cost and higher convenience. All primers were tested for efficiency by serial dilutions and specificity by melting peak analysis. RT was performed on a ABI-9600 with reagents from New England Biolabs (USA). qPCR was performed in technical quadruplicates on an ABI-7900HT Sequence Detection System equipped with a 384-well block (Applied Biosystems, USA). The biological sample sizes were as indicated in the figure legends. Data were analyzed using SDS 2.3 software (Applied Biosystems, USA) and Microsoft Excel 2011 software (Microsoft Corp, USA). Relative quantification and the DCq method were used. For quantification of miRNAs, normalization was performed relative to the corresponding levels of miR-451a, which previously showed no significant change following treatment.

STATISTICS

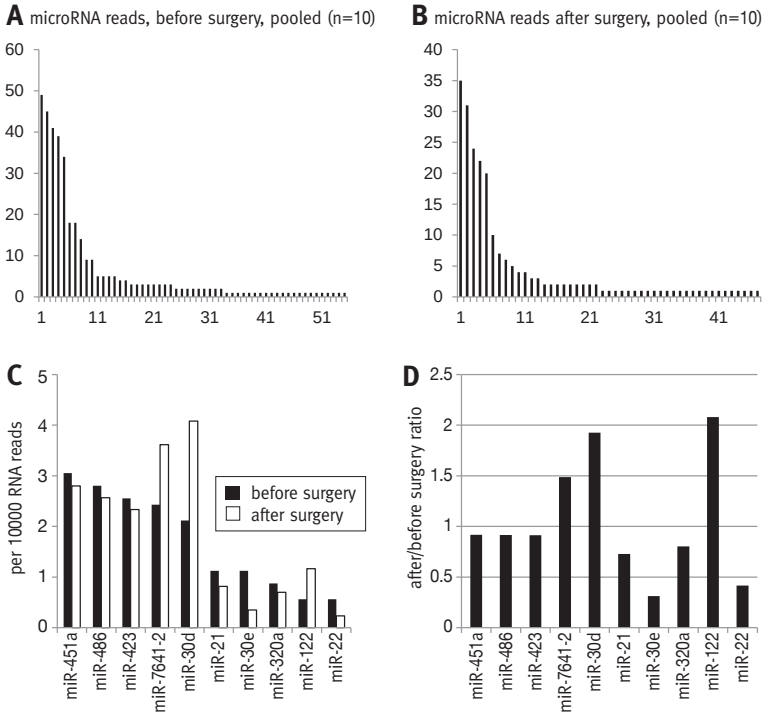
For statistical tests, Student's *t*-test was used. For correlations, linear regression was used.

RESULTS

miR-122 levels were elevated in sera of patients whose endothelial function had improved 3 months following bariatric surgery

To identify miRNA candidates that were affected by bariatric surgery, RNA samples before and after surgery from 10 patients (out of the total of 21) who showed the biggest increases in FMD, were combined into two pools, which were profiled by miSeq sequencing. miRbase-annotated miRNAs were represented only in a minute fraction of the reads (0.2%). Although approximately 50 miRNAs could be identified in both pools, only a part of these had enough counts to be quantitatively compared between the pools [Figures 1A, 1B]. We focused on the 10 highest-expressed miRNAs. Of these, the levels of several miRNAs differed between the two pools. Thus, miR-7641-2, miR-30d, and miR-122 were up-regulated after surgery, while miR-21, miR-30e, and miR-22 were down-regulated [Figures 1C, 1D].

Figure 1. miSeq profiling of miRNAs in pooled sera from 10 patients, before and 3 months after bariatric surgery. **[A]** distribution of miRbase reads in pooled samples before surgery, **[B]** relative levels for all miRNAs that passed QC, before and after surgery. Read counts were normalized to the total number of small RNA reads. Log scale, **[C]** normalized reads for 10 most abundant miRNAs, before and after surgery, **[D]** before/after surgery ratios of reads for 10 most abundant miRNAs



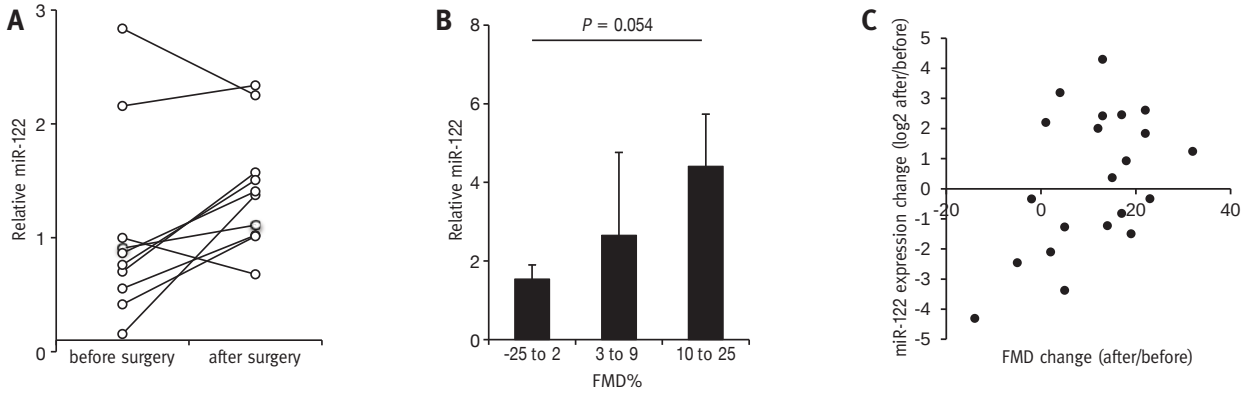
miRNAs = MicroRNAs

To validate these findings, levels of candidate miRNAs were measured by QRT-PCR in the individual samples that were used for the pools (7 males, 3 females, paired samples before and 3 months after surgery), on a ABI-7900HT Sequence Detection System station, and using a primer extension method as described by Balcells and colleagues [19]. Expression levels were normalized to miR-451a, which showed consistent levels across patients and pre/post-surgery samples. Of the miRNAs tested, miR-122 was up-regulated post-surgery as compared to pre-surgery levels [Figure 2A]. In our sample, this up-regulation occurred in 8 out of 10 patients and was significant ($P = 0.025$, paired t-test).

Serum miR-122 levels correlate positively with endothelial function as measured by FMD and the serum miR-122/miR-451 ratio may serve as a marker for endothelial function in obese patients

To check if serum miR-122 levels correlate positively with endothelial function, miR-122 and miR-451a (for normalization) were measured in sera from 21 bariatric surgery patients before and 3 months post-surgery (a total of 42 samples with corresponding FMD values). This experiment had no FMD criteria and included the 10 patients mentioned earlier. The results were grouped into 3 bins of 14 samples each, based on FMD ranges: low (-25% to 2%), medium (3% to 9%), and high (10% to 25%). miR-122 levels (normalized to miR-451a) tended to correlate with FMD values, with a borderline significant ($P = 0.054$) three-fold difference in miR-122 levels between the high and low FMD bins [Figure 2B]. The changes in miR-122 levels (normalized to miR-451a) from pre- to 3 months post-surgery also tended to correlate with the respective changes in FMD [Figure 2C].

Figure 2. QRT-PCR for miR-122 in individual serum samples. **[A]** relative miR-122 levels in 10 patients with the most FMD increases as a result of the surgery (7 male, 3 female), before and after surgery. Levels were normalized to miR-451a. Note logarithmic scale, **[B]** relative miR-122 levels in 21 patients, including the 10 included in **[A]**, both before and after surgery (total of 42 samples), subdivided by FMD into 3 groups of 14 samples each. Levels were normalized to miR-451a. Bars, standard error. Student's t-test between highest and lowest FMD groups showed borderline significance ($P = 0.054$), **[C]** individual pre-/post-surgery changes of miR-122 levels plotted against the corresponding individual FMD changes following the surgery. Levels were normalized to miR-451a



QRT-PCR = quantitative reverse transcription-polymerase chain reaction, FMD = flow mediated dilation

DISCUSSION

Bariatric surgery helps people reduce body weight and thus improves diabetes, hyperlipidemia, hypertension, and obstructive sleep apnea [2,3]. Long-term outcomes of surgically induced weight loss (the Swedish Obese Subjects study [3-5]) demonstrated that long-term sustained weight reduction also reduced the incidence of diabetes, cardiovascular events, and cancer. Our results show that the levels of miR-122, the dominant liver miRNA with a known function in lipid metabolism [20], are elevated in the sera of patients 3 months after bariatric surgery, and that this elevation is broadly correlated to the improvement in endothelial function in these patients. Prior studies have shown elevated levels of circulating miR-122 in obesity [21] and suppression of miR-122 following bariatric surgery [22], as well as differential changes in the levels of this miRNA in the liver and other organs [23]. These results may be reconciled when the dynamics of post-surgery homeostatic adjustments are taken into account. Thus, the levels of miR-122 in the blood are likely to represent a dynamic equilibrium with its levels in tissues, primarily the liver. In obesity, high adiposity levels in the liver, accompanied by a persistent inflammatory state, likely lead to somewhat elevated miR-122 secretion into the blood. Bariatric surgery leads to an adjustment in this equilibrium. Secretion of lipids stored in the liver also liberates large quantities of miR-122 into the bloodstream, as we detected 3 months post-surgery, when weight loss is rapid as is overall metabolic readjustment. It is also likely that at later times after surgery, blood miR-122 levels decrease once more, as an indication of a newly formed post-surgery metabolic equilibrium. In addition, most recent studies describe the newer Roux-en-Y (RYGB) bypass surgery, while our patients underwent the gastric sleeve operation. The different surgical approach may explain the discrepancy between our findings (up-regulation of miR-122 following the sleeve operation) and prior studies, showing down-regulation of miR-122 after bariatric surgery. It has been shown that RYGB surgery, but not gastric sleeve surgery, increased circulating bile acids and TGR5 signaling [24], supporting the notion that different surgical methods may indeed cause different clinical metabolic consequences.

The ability of miR-122 to suppress tumorigenesis when delivered to a non-inflammatory *MYC*-driven hepatocellular carcinoma model established the idea that miR-122 could perform a tumor suppressor function independently of its role in reducing inflammation [25]. Reduced miR-122 expression in hepatocellular carcinoma correlates with metastasis and poor prognosis. Deletion of murine miR-122 resulted in hepatosteatosis, hepatitis, and the development of tumors resembling hepatocellular carcinoma. Delivery of miR-122 to a *MYC*-driven mouse model of hepatocellular carcinoma strongly inhibited tumorigenesis, further supporting the tumor suppressor activity of this miRNA [25]. Additional studies are under way to elucidate the mechanism of its secretion into circulation and its absorption by VECs, as well as its relevant cellular targets.

LIMITATIONS OF THE STUDY

The small sample size may have caused a beta error in the statistical analysis, and should guide us to continue this study in larger populations. The fact that measurements were done in one time point is another limitation, and should be expanded to several time points so that levels of miRNA will be studied in longer periods of time following the bariatric surgery. It could be that a longer follow-up would have shown down-regulation of miR-122 following the increase that was observed 3 months post-surgery.

Funding

This study was funded by the Gesher Award from the Israel Cancer Research Fund (ICRF) and the Ministry of Science and Technology, Israel; by a Diabetes Research Grant from D-Cure and the Ministry of Health, Israel, to AM; and by the European Union's CEREHA project (FP7-REGPOT-2012-2013-1, Agreement No 316157).

Correspondence

Dr. A. Meerson

Molecular Biology of Chronic Diseases Laboratory, MIGAL Galilee Research Institute, Kiryat Shmona 11016, Israel

Fax: (972-4) 665-2687

email: Arim@migal.org.il

References

1. Hjartåker A, Langseth H, Weiderpass E. Obesity and diabetes epidemics: cancer repercussions. *Adv Exp Med Biol* 2008; 630: 72-93.
2. Tee MC, Cao Y, Warnock GL, Hu FB, Chavarro JE. Effect of bariatric surgery on oncologic outcomes: a systematic review and meta-analysis. *Surg Endosc* 2013; 27: 4449-56.
3. Sjöström L, Larsson B, Backman L, et al. Swedish obese subjects (SOS). Recruitment for an intervention study and a selected description of the obese state. *Int J Obes Relat Metab Disord* 1992;16 (6): 465-79.
4. Sjöström L, Lindroos AK, Peltonen M, et al. Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. *N Engl J Med* 2004; 351 (26): 2683-93.
5. Monte SV, Caruana JA, Ghanim H, et al. Reduction in endotoxemia, oxidative and inflammatory stress, and insulin resistance after Roux-en-Y gastric bypass surgery in patients with morbid obesity and type 2 diabetes mellitus. *Surgery* 2012; 151 (4): 587-93.
6. Vázquez LA, Pazos F, Berrazueta JR, et al. Effects of changes in body weight and insulin resistance on inflammation and endothelial function in morbid obesity after bariatric surgery. *J Clin Endocrinol Metab* 2005; 90: 316-22.
7. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; 120: 15-20.
8. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 2007; 27: 91-105.
9. Friedman Y, Balaga O, Linial M. Working together: combinatorial regulation by microRNAs. *Adv Exp Med Biol 2013;* 774: 317-37.
10. Fernandez-Hernando C, Suarez Y, Rayner KJ, Moore KJ. MicroRNAs in lipid metabolism. *Curr Opin Lipidol* 2011; 22: 86-92.
11. Heneghan HM, Miller N, Kerin MJ. Role of microRNAs in obesity and the metabolic syndrome. *Obes Rev Off J Int Assoc Study Obes* 2010; 11: 354-61.
12. Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes* 2009; 58: 1050-7.
13. Viesti A, Collares R, Salgado W Jr, Pretti da Cunha Tirapelli D, dos Santos JS. The expression of LEP, LEPR, IGF1 and IL10 in obesity and the relationship with microRNAs. *PLoS One* 2014; 9 (4): e93512.

14. Dhahbi JM, Spindler SR, Atamna H, et al. 5' tRNA halves are present as abundant complexes in serum, concentrated in blood cells, and modulated by aging and calorie restriction. *BMC Genomics 2013;* 14: 298.

15. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem 2010;* 56: 1733-41.

16. Shifeng H, Danni W, Pu C, Ping Y, Ju C, Liping Z. Circulating liver-specific miR-122 as a novel potential biomarker for diagnosis of cholestatic liver injury. *PLoS One* 2013; 8 (9): e73133.

17. Wang B, Wang H. Yang Z. MiR-122 inhibits cell proliferation and tumorigenesis of breast cancer by targeting IGF1R. *PloS One* 2012; 7 (10): e47053.

18. Basu S, Bhattacharyya SN. Insulin-like growth factor-1 prevents miR-122 production in neighbouring cells to curtail its intercellular transfer to ensure proliferation of human hepatoma cells. *Nucleic Acids Res* 2014; 42: 7170-85.

19. Balcells I, Cirera S, Busk PK. Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. *BMC Biotechnol* 2011; 11: 70.

20. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Meta 2006;* 3 (2): 87-98.

21. Gao W, He HW, Wang ZM. et al. Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. *Lipids Health Dis* 2012; 11: 55.

22. Ortega FJ, Mercader JM, Catalán V, et al. Targeting the circulating microRNA signature of obesity. *Clin Chem 2013;* 59: 781-92.

23. Kwon IG, Ha TK, Ryu SW, Ha E. Roux-en-Y gastric bypass stimulates hypothalamic miR-122 and inhibits cardiac and hepatic miR-122 expressions. *J Surg Res 2015;* 199: 371-7.

24. Kohli R, Bradley D, Setchell KD, Eagon JC, Abumrad N, Klein S. Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids. *J Clin Endocrinol Metab* 2013; 98 (4): E708-12.

25. Hsu SH, Wang B, Kota J, et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest 2012;* 122 (80): 2871-83.

Capsule

Local macrophage clean-up

Infection, especially by helminths or bacteria, can cause tissue damage. Minutti and colleagues studied mouse models of helminth infection and fibrosis. They expressed surfactant protein A (a member of the complement component C1q family) in the lung, which enhanced interleukin-4 (IL-4)-mediated proliferation and activation of alveolar macrophages. This activation accelerated helminth clearance and reduced lung injury. In the peritoneum, C1q boosted macrophage activation for liver repair after bacterial infection. By a different approach, Bosurgi and co-authors discovered that after wounding caused by migrating helminths in the lung or during inflammation in the gut of mice, IL-4 and IL-13 act only in the presence of apoptotic cells to promote tissue repair by local macrophages.

Science 2017; 356: 1076 (Minutti), 1072 (Bosurgi)

Eitan Israeli

Capsule

Old cancer drugs with a modern mechanism

Some cancer drugs are rationally designed on the basis of their known interaction with specific target molecules that drive tumorigenesis. Others are mechanistically poorly understood but are developed because they display anti-cancer activity with low toxicity in preclinical models. Uehara et al. identified the mechanism underlying the anti-cancer activity of a class of drugs in the latter category—the sulfonamides. They found that three different sulfonamides (E7820, indisulam, and CQS) induce formation of a complex between a specific RNA-splicing factor and a specific E3 ubiquitin ligase. This interaction promotes selective degradation of the splicing factor. Interestingly, selective protein degradation also explains the activity of an unrelated cancer drug called lenalidomide.

Nat Chem Biol 2017; 10.1038/nchembio.2363

Eitan Israeli

Capsule

The NET effect of viral-triggered asthma

Infection with rhinovirus is a common cause of allergic asthma. Toussaint and co-authors studied how the virus triggers inflammation and stimulates an asthmatic attack. Rhinovirus infection causes the release of host double-stranded DNA and the formation of neutrophil extracellular traps (NETs). NETs are structures that capture microorganisms and activate immune cells and inflammatory responses. The authors showed that rhinovirus-driven NETs promote the infiltration of inflammatory cells to the airways, causing the clinical features of an allergic response. Treatment with a compound blocking NET formation stopped the asthma from becoming worse.

Nat Med 2017; 10.1038/nm.4332

Eitan Israeli